CHARACTERIZATION OF THE VASCULAR CONTROL OF HINDLIMB METABOLISM

by

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PREFACE

The majority of the experimental work presented in this thesis was carried out in the period from February 1990 to January 1993. Work performed by other members of this department has been presented and acknowledged where relevant. In this regard, contributions were made by Dr Stephen Rattigan in the insulin-mediated glucose uptake studies (Chapter 5), Dr Steve Edwards in the microsphere studies (Chapter 7), and Mr John Newman in the fluorescent marker entrapment studies (Chapter 7).

Some of the data presented here has been published or presented at scientific meetings and the relevant publications are listed below.

Publications directly arising from this thesis:

1. Dora, K.A., Colquhoun, E.Q., Hettiarachchi, M., Rattigan, S. and Clark, M.G. (1991) The apparent absence of serotonin-mediated vascular thermogenesis in perfused rat hindlimb may result from vascular shunting. *Life Sci.* **48**: 1555-1564.

2. Dora, K.A., Richards, S.M., Rattigan, S., Colquhoun, E.Q. and Clark, M.G. (1992) Serotonin and norepinephrine-mediated vasoconstriction in the perfused rat hindlimb have different oxygen requirements. *Am. J. Physiol.* **262**: H698-H703.

Currently unpublished papers directly arising from this thesis:

3. Rattigan, S., Dora, K.A., Colquhoun, E.Q. and Clark, M.G. (accepted subject to revision) Serotonin-mediated acute insulin resistance in the perfused rat hindlimb but not in incubated muscle: a role for the vascular system. *Life Sci.*

4. Dora, K.A., Rattigan, S., Colquhoun, E.Q. and Clark, M.G. (submitted) Serotonin impairs aerobic muscle contraction in perfused rat hindlimb. *J. Appl. Physiol.*

Additional publications:

5. Richards, S.M., Dora, K.A., Hettiarachchi, M., Rattigan, S. and Colquhoun, E.Q. and Clark, M.G. (1992) A close association between vasoconstrictor-mediated uracil and lactate release by the perfused rat hindlimb. *Gen. Pharmacol.* **23**: 65-69.

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9. Matthias, A., Richards, S.M., Dora, K.A., Clark, M.G., and Colquhoun, E.Q. (accepted subject to revision) Characterization of perfused periaortic brown adipose tissue from the rat. *Can. J. Physiol. Pharmacol.*

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11. Richards, S.M., Clark, M.G., Steen, J.T., Dora, K.A., Rattigan, S., and Colquhoun, E.Q. (submitted) Release of purines and pyrimidines from perfused rat hindlimb, perfused mesenteric arcade and incubated de-endotheliated aorta. *J. Vasc. Res.*

12. Clark, M. G., Colquhoun, E.Q., Dora, K.A., Rattigan, S., Eldershaw T.P.D., Hall J.L. and Ye J-M. (submitted) Resting muscle: a source of thermogenesis controlled by vasomodulators. **In**: *Temperature Regulation*, edited by A.S. Milton. UK: Birkhauser.

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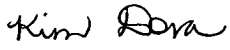
STATEMENT

This thesis contains no material which has been used for the award of any other higher degree or graduate diploma in any tertiary institution without written permission and, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.



Kim Dora

ABBREVIATIONS

5-HT	5-Hydroxytryptamine, serotonin
5-MU	5-Methyl urapidil
ADP	Adenosine diphosphate
AR	Adrenoceptor
ATP	Adenosine triphosphate
AVA	Arteriovenous anastomoses
BSA	Bovine serum albumin
cAMP	Adenosine 3',5'-cyclic phosphate
CEC	Chloroethylclonidine
cGMP	Guanosine 3',5'-cyclic phosphate
CrP	Creatine phosphate
DAG	1,2-Diacylglycerol
EDL	Extensor digitorum longus
EDRF	Endothelium-derived relaxing factor
EGTA	Ethyleneglycol-bis-(b-aminoethyl ether) N,N,N',N'-tetraacetic acid
FITC	Fluorescein isothiocyanate
IP_3	Inositol 1,4,5-triphosphate
NAD	Nicotine adenine dinucleotide
NE	Norepinephrine, noradrenaline
NIDDM	Non-insulin-dependent diabetes mellitus
PO_2	Oxygen partial pressure
RBC	Red blood cell
SHR	Spontaneously hypertensive rat
SR	Sarcoplasmic reticulum
UTP	Uridine triphosphate
$\dot{V}O_2$	Oxygen uptake

ABSTRACT

Characterization of the vascular control of hindlimb metabolism

The constant-flow perfused rat hindlimb was used as a model to characterize norepinephrine (NE, noradrenaline)- and serotonin (5-HT)-mediated vasoconstriction and their associated effects on metabolism. Rat hindlimbs of various sizes were perfused using medium with or without erythrocytes at temperatures varying between 25 and 37°C. Varying these perfusion parameters altered the quantitative, but not qualitative, effects of the hormones.

In the hindlimb, infusion of NE stimulated both α_1- and β-adrenoceptors (AR). The overall effect was the rapid onset of a sustained increase in perfusion pressure which was only slightly increased upon β-AR blockade. Energy for NE-stimulated vasoconstriction was supplied via aerobic means, involving oxidative formation of ATP. In addition, vasoconstriction appeared to be dependent on extracellular Ca^{2+}, suggesting an α_{1A}-AR-stimulated Ca^{2+} influx through plasma membrane channels.

In association with vasoconstriction, NE dose-dependently increased hindlimb oxygen uptake ($\dot{V}O_2$) and lactate release via stimulation of α_1-AR. During prolonged NE infusion, lactate release included a minor contribution by β-AR-stimulated glycogenolysis. NE impaired insulin-mediated glucose uptake via stimulation of β-ARs.

Infusion of 5-HT dose-dependently caused sustained increases in hindlimb perfusion pressure by stimulation of 5-HT_{2A}- and 5-HT_1-like receptors. Upon blockade of these receptors, a vasodilatory component was unmasked, presumably via stimulation of orphan 5-HT-receptors. Vasoconstriction by 5-HT and supra-physiological high-doses of NE, the latter acting through a subgroup of α_1-AR, could be supported through either aerobic and/or anaerobic metabolism and was not extracellular Ca^{2+}-dependent. In Ca^{2+}-free conditions, NE stimulated direct inhibition of $\dot{V}O_2$ in association with markedly attenuated rises in perfusion pressure, consistent with stimulation of α_{1B}-ARs.

In association with vasoconstriction, both 5-HT and high-dose NE decreased hindlimb $\dot{V}O_2$. 5-HT inhibited, whereas high-dose NE had no effect, on basal lactate release. Both agonists impaired hindlimb insulin-mediated glucose uptake, the NE

impairment involving stimulation of both α_1- and β-ARs. In addition, infusion of 5-HT impaired sciatic nerve-stimulated striated muscle aerobic contraction in perfused, but not incubated, preparations.

In contrast to the observations in the hindlimb, both 5-HT and NE increased $\dot{V}O_2$ and lactate release in association with vasoconstriction in the isolated, constant-flow perfused mesenteric artery arcade.

These results, in conjunction with literature reports, suggest NE and 5-HT control hindlimb metabolism by site-selective vasoconstriction. Lower doses of NE stimulated α_1-AR probably located on arterioles within the microcirculation, increased vascular smooth muscle metabolism, and may also have redistributed flow to highly metabolically active regions. Conversely, 5-HT_2-receptors and a separate subgroup of α_1-AR are proposed to be located at discrete vascular sites on larger blood vessels that, when stimulated, selectively redistributed blood-flow (functional vascular shunting), resulting in reduction of oxygen and glucose delivery to metabolically active hindlimb tissues.

Thus the control of blood flow and distribution by endogenous vasoactive agents alters tissue metabolism. Further characterization of these effects and the relevance to *in vivo* situations are discussed and may give important insights into the causes, treatment and links between some aspects of obesity, hypertension and type II diabetes.

CHAPTER 1

Thesis introduction

1.1 Control of blood flow in skeletal muscle.

The expenditure of energy may be related to a state of rest or to different degrees of physical exertion. Resting energy expenditure (which is usually measured under standardized conditions) is a complex function of the unavoidable thermal loss associated with cellular metabolism and physiological functions sustained at repose. The skeletal muscle mass comprises approximately 40% of total body mass in man (Dickerson & Widdowson, 1960), and accounts for over 25% of the total oxygen uptake ($\dot{V}O_2$) in resting humans (Brozek & Grande, 1955), and up to 90 percent during intense exercise (Lehninger, 1982). Alterations in skeletal muscle metabolism contribute to the problems associated with a number of disorders including obesity (Zurlo *et al.*, 1990) and diabetes (Klip & Marette, 1992).

Blood flow in skeletal muscle serves to supply nutrients to the muscle fibres and to remove metabolites; it influences tissue growth and atrophy, and affects the response of muscle in disease. In the 1920s Krogh (cited in Krogh, 1959) reported the flow through an organ was variable due to local contractions and dilatations, and he considered this an important contribution to maintaining "uniformity of the circulation". Indeed, this observation arguably led to the large body of research investigating the central, neural and local vasoactive agents controlling tissue nutrient supply in accordance with demand.

1.1.1 Vasoactive agents.

The circulation has an intricate system for regulation of arterial pressure. Briefly, normal mean arterial pressure is maintained by both rapid onset nervous and hormonal mechanisms and by slow onset kidney function and blood volume regulation mechanisms. The central nervous system controls blood pressure and blood flow, by regulating vascular smooth muscle tone, and cardiac rate and contractility.

Increases in sympathetic nervous system activity, induced by situations including feeding, cold and hypoxia (Appenzeller, 1990), are modulated by arterial

baroreceptor and chemoreceptor reflexes (Spyer, 1990). The exquisite control that the sympathetic nervous system exerts is achieved by the interaction of variable innervation and numerous differences in transmission characteristics and effector cell sensitivity. The major sympathetic neurotransmitter released at the effector organs is norepinephrine (NE), which stimulates a host of responses including vasoconstriction through post-synaptic α_1- and α_2-adrenoceptors (AR). All blood vessels are innervated by sympathetic fibres (Mulvany & Aalkjær, 1990). The number of adrenergic nerve terminals and receptors increases with decreasing vessel size, with the highest density surrounding the small arteries and arterioles (Fuxe & Sedvall, 1965; Nilsson *et al.*, 1986; Hirst & Edwards, 1989). Other co-transmitters such as ATP and neuropeptide Y may induce vasoconstriction independently, but generally amplify the effects of NE (Pernow *et al.*, 1986; Burnstock, 1990). Circulating serotonin (5-HT) has been shown to amplify sympathetic and NE-mediated vasoconstriction within vessels of hindlimbs (Medgett, 1987; Van Nueten & Janssens, 1988; Szabó *et al.*, 1991), and may be taken up and stored in sympathetic nerve endings (reviewed by Urabe *et al.*, 1991).

NE enters plasma principally by overflow of released neurotransmitter to the circulation (reviewed by Esler *et al.*, 1990), and resting skeletal muscle contributes approx. 20% of plasma NE (Esler *et al.*, 1984). 5-HT released by central or peripheral nerves and by chromaffin cells also overflows to the plasma. In many instances plasma levels of NE and 5-HT are low due to the rapid uptake by circulating platelets and endothelial cells, particularly in the pulmonary artery (Bhat & Block 1990). Thus the circulating free concentration of NE (reviewed by Esler *et al.*, 1990) and 5-HT (Vanhoutte & Cohen, 1983) are usually very low. In addition to uptake, platelets store and release bioamines such as 5-HT, histamine, epinephrine and NE (Da Prada *et al.*, 1981). The amounts of 5-HT released from aggregating platelets are sufficient to contract the blood vessel wall *in vitro* (reviewed by Vanhoutte & Cohen, 1983).

In addition to vasoconstriction (via stimulation of 5-HT_2 receptor subtypes), 5-HT also stimulates vasodilatation and a host of other responses by stimulation of various receptor subtypes (Humphrey *et al.*, 1993).[1] Thus 5-HT can lead to complex

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1. 5-HT receptor nomenclature has recently been modified from the previously accepted Bradley *et al.* nomenclature described in 1986 (in Humphrey *et al.*, 1993). Changes relevant to this thesis are 5-HT_{2A} for 5-HT_2; 5-HT_{2C} for 5-HT_{1C}; 5-HT_{1C}-like for the non 5-HT_{1A}-, 5HT_{1B}-, or 5-HT_{1D}-receptor negatively coupled to

changes in the cardiovascular system which may comprise bradycardia or tachycardia, and hypotension or hypertension. The eventual response depends, among other factors, upon the species, the vascular bed under study, and the dose employed (reviewed by Saxena & Villalón, 1990). 5-HT can vasodilate blood vessels either directly by stimulation of 5-HT_1-receptor subtypes, or indirectly by inhibiting NE release from sympathetic nerve terminals, and releasing EDRF (Mylecharane, 1990). An area receiving current attention is the control of arteriovenous-anastomoses (AVAs) blood flow in certain vascular beds. In the human forearm, porcine hindleg and carotid artery, 5-HT appears to improve nutrient blood flow by vasoconstriction through 5-HT_1-like receptors and a resulting reduction of flow through AVAs (Blauw *et al.*, 1991; den Boer *et al.*, 1991). One spin-off has been that 5-HT_1-like receptor stimulation improved cerebral flow and could treat migraine (Ferrari & Saxena, 1993).

Other important humoral factors that affect circulatory function include the potent vasoconstrictors angiotensin II and vasopressin (reviewed by Berecek & Swords, 1990). Renin released from the kidneys is converted to angiotensin II by angiotensin converting enzyme located in blood vessels. Angiotensin II stimulates AT_1-receptors located in most blood vessels. Vasopressin is released from the posterior pituitary gland to the blood. Normally plasma levels are low, but may rise during haemorrhage. Vasopressin stimulates V_1-receptors to stimulate vasoconstriction and V_2-receptors in the kidney to retain water.

In addition to the actions of neuronally released or circulating vasoactive agents, vascular smooth muscle may constrict or dilate in response to raised or lowered intravascular pressure, respectively, termed the myogenic response, leading to autoregulation of flow (initially proposed by Bayliss, 1902). These phenomena have also been reported within resting skeletal muscle (Stainsby & Renkin, 1961; Grände *et al.*, 1977). More recently it has been shown that locally released vasodilators (endothelium-derived relaxing factors, EDRFs) provide an opposing feedback mechanism to confer overall stability (Griffith *et al.*, 1987; Bevan & Joyce, 1990; Smieško & Johnson, 1993).

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adenylate cyclase; and orphan 5-HT-receptor for the receptor mediating direct vasodilatory effects through 5-HT. This current recommended terminology will be used throughout for consistency, regardless of terminology used by the authors of articles cited.

1.1.1.1 Involvement in thermoregulation.

The maintenance of body temperature in health and disease is one of the fundamental physiological functions of the homeotherm (reviewed by Cassel & Casselman, 1990). During thermoregulation, the multiple levels of reception and control in conjunction with the segmental arrangements of the sympathetic nervous system permit both general and highly specific responses (Riedel & Iriki, 1979).

Hsieh *et al.* (1957) demonstrated that in mice exposed to cold, sympathetic nervous system activity, through NE, increased body temperature without shivering. This has now been demonstrated in many species, including humans. Plasma levels of NE increase in proportion to degrees of cold exposure, due to discriminant release from organs or tissues, including skeletal muscle (reviewed by Landsberg & Young, 1983). The release of catecholamines from the adrenal medulla also plays a role in cold-acclimation (reviewed by Landsberg & Young, 1983).

In humans, skin sympathetic nerve activity is involved primarily in thermoregulation, and decreases with increases in body temperature (Wallin & Fagius, 1988). The "cold-pressor test" (Fagius *et al.*, 1989) and cold-exposure (Moore *et al.*, 1972) induce rises in blood pressure, each presumably due to increased muscle sympathetic nerve activity (Fagius *et al.*, 1989).

1.1.1.2 Release postprandially.

It is widely accepted that caloric restriction suppresses, while increased caloric intake stimulates, the sympathetic nervous system, thereby altering cardiovascular function, including blood flow distribution (reviewed by Landsberg & Young, 1983). After ingestion of protein, fat or carbohydrate an increased $\dot{V}O_2$ has been observed in the postprandial period (reviewed by Landsberg & Young, 1983).

Fagius and his co-workers have used microneurography to measure impulses by sympathetic nerves in human extremity nerves (Wallin & Fagius, 1988). Using this technique they have found that insulin-induced hypoglycaemia (Fagius *et al.*, 1986) and infusion of 2-deoxy-D-glucose (Fagius & Berne, 1989) increased skeletal muscle sympathetic nerve activity to resistance vessels. These effects were consequences of central nervous system glucopaenia, and not the previously suggested baroreceptor-induced counteraction of cardiovascular changes (reviewed by Mano, 1990). In

addition to increased sympathetic nervous system activity, infusion of glucose (Welle *et al.*, 1981; Levin, 1991), but not protein (Welle *et al.*, 1981) into rats raised plasma NE levels. However, NE did not appear to affect glucose levels when infused into dogs (except at very high NE levels, possibly due to hepatic glucose production, Connolly *et al.*, 1991), suggesting NE *per se* does not have a role in glucose disposal and has an alternative role when released upon glucose infusion. NE release from skeletal muscle sympathetic nerve endings would alter and perhaps control blood flow, and may also increase metabolism in this tissue by vasoconstriction-associated consumption of energy (Colquhoun & Clark, 1991).

In genetically obese Zucker rats, which exhibit defective thermogenic capacity and hyperinsulinaemia, NE biosynthesis is reduced (particularly in brown adipose tissue) leading to lower free NE levels upon sympathetic nervous system activation (Levin *et al.*, 1983).

1.1.1.3 Release during exercise.

Increased metabolic demand associated with skeletal muscle work *in vivo* causes an increase in flow, and thereby oxygen, nutrient and metabolite passage, in proportion to the intensity of exercise. The communication among endothelial and smooth muscle cells normally causes vasodilatation and thereby hyperaemia to the exercising muscle by a combination of flow-dependent and endothelial cell-mediated relaxation (reviewed in Segal, 1992).

There is a direct relationship between muscle blood flow and slow-twitch oxidative fibre populations (Laughlin & Armstrong, 1982), with α-AR stimulation limiting flow to fast-twitch glycolytic fibres (Laughlin & Armstrong, 1987). Exercise-mediated NE spillover from sympathetic nerves has been reported in humans (Hasking *et al.*, 1988) and dogs (Peronnet *et al.*, 1988). Exercise-stimulated sympathetic nervous system activity acts predominantly to redistribute cardiac output away from inactive areas. However, as exercise intensity increases, the maximal flow to contracting muscle can be limited by sympathetic vasoconstriction (Joyner *et al.*, 1990, 1992).

Several studies have shown that changes in blood flow can alter the force exerted by a muscle contracting intermittently during an extended period. This has been demonstrated in normal man (Barcroft & Millen, 1939), in man with cardiac

impairment (Donald *et al.*, 1957) and in the isolated cat gastrocnemius-soleus (Hirvonen & Sonnenschein, 1962; Wright & Sonnenschein 1965).

1.1.1.4 Action during hypoxia.

Regional hypoxia has been shown to stimulate the release of the vasodilators prostaglandin I_2 (Busse *et al.*, 1984) and EDRF (Pohl & Busse, 1989). Under anoxic conditions, EDRF is no longer produced (De Mey & Vanhoutte, 1983) and an endothelium-derived constricting factor is released (Rubanyi & Vanhoutte, 1985). During systemic hypoxia, flow is diverted away from nutritive capillaries without compromising tissue need (Harrison *et al.*, 1990b) by altering the locus of myogenic tone to larger blood vessels (Bertuglia *et al.*, 1991), predominantly by increased sympathetic nerve activity (Mian & Marshall, 1991a).

In general, larger vessels are usually able to maintain constriction during hypoxic conditions (Shibata & Briggs, 1967; Altura & Altura, 1976; De Mey & Vanhoutte, 1983). Conversely, small blood vessels tend to dilate in response to low oxygen tensions (Carrier *et al.*, 1964; Duling, 1972; Harris *et al.*, 1976).

1.1.1.5 Contribution to disease states.

Changes in the flow to organs or tissues have profound implications for nutrient and hormone delivery as well as product removal. Disorders such as hypertension, obesity and Type II, non-insulin-dependent diabetes mellitus (NIDDM) are usually associated with increased sympathetic nervous system activity (reviewed by Daly & Landsberg, 1991), vascular disease (reviewed by Ruderman *et al.*, 1992) and altered responsiveness to vasoactive agents (reviewed by Struyker-Boudier *et al.*, 1990). Interestingly, low capillary density in skeletal muscle has been correlated with increased fat deposition and insulin-resistance (Lillioja *et al.*, 1987). In addition, modifications exist in the hindlimb skeletal muscle vasculature of spontaneously hypertensive rats (SHR) that reduce the capillary exchange capacity and limit flow delivery (Sexton *et al.*, 1990).

There is growing clinical (reviewed by Julius *et al.*, 1992) and experimental (Hwang *et al.*, 1987; Mondon & Reaven, 1988; Finch *et al.*, 1990) evidence that hypertension and NIDDM are often associated with each other, and the occurrence of one may lead to the symptoms of another. Insulin resistance and hyperinsulinaemia

are commonly associated with hypertension in the obese, particularly upper body "abdominal" obesity (Landsberg, 1990). These associations have led Landsberg and co-workers to propose that the insulin resistance of obesity, and its consequent hyperinsulinaemia, stimulates the sympathetic nervous system, which then drives thermogenic mechanisms, thereby increasing metabolic rate and limiting further weight gain. The hyperinsulinaemia and sympathetic stimulation, via effects imposed on the kidney, heart and blood vessels, result in hypertension (reviewed in Landsberg & Krieger, 1989). This hypothesis was extended by the proposal that diet-induced increases in sympathetic activity to muscle vasculature (Fagius & Berne, 1989) may contribute to the whole body increase in metabolic rate by consuming energy during vasoconstriction (Clark *et al.*, 1991; Figure 1.1).

However it is not clear whether the link between hypertension and insulin resistance is indicative of a causal relationship as proposed by Landsberg and Krieger (1989). The relationship between cause and effect remains unclear for humans as well as animal models (Buchanan *et al.*, 1992; Bursztyn *et al.*, 1992; Mitrakau *et al.*, 1992). Indeed, there is accumulating clinical evidence demonstrating the importance of blood flow control to skeletal muscle in insulin-resistant patients with hypertension (reviewed by Julius *et al.*, 1992). Evidence supporting a haemodynamic basis for the insulin resistance observed in patients with elevated mean arterial pressure (and presumably hypertension) is provided by Baron *et al.* (1993, and references therein) who showed that flow was reduced to skeletal muscle, thereby impairing the delivery of insulin and glucose and creating a state of insulin resistance. Microvascular rarefaction observed in the microcirculation of skeletal muscle in SHRs (Prewitt *et al.*, 1982; le Noble *et al.*, 1990) and human essential hypertension (Henrich *et al.*, 1988) contributes to the rise in blood pressure and may further decrease the delivery of nutrients and hormones to the muscle. Recent evidence (Saltzman *et al.*, 1992) suggests that within skeletal muscle of SHRs, adrenergic nerve plexus density increases to the majority of vessels, possibly contributing to elevated arteriolar tone.

In addition to alterations in sympathetic nervous system activity, certain disease states are associated with changes in levels of circulating vasoactive agents. Increased plasma levels of 5-HT have been observed in patients with hypertension (reviewed by Vanhoutte, 1982), NIDDM (Barradas *et al.*, 1988) and other diseases including atherosclerosis (reviewed by Barradas & Mikhailidis, 1992).

In hypertension, decreases in blood vessel internal (hypertrophy) and external (remodelling) diameters impair maximal vasodilatation (reviewed by Heistad *et al.*,

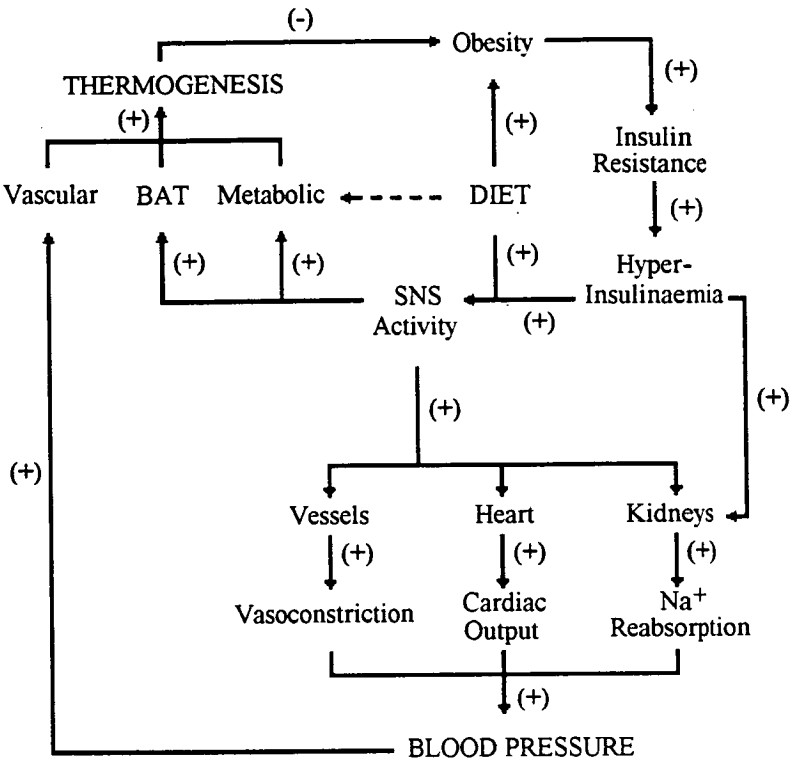

Figure 1.1 Modified Landsberg hypothesis.

This modification depicts the proposed relationship between obesity, insulin, sympathetic nervous system (SNS) activity, blood pressure and vascular thermogenesis. Blood pressure is shown as a positive stimulus for increased vascular thermogenesis which is a component of the total thermogenic response along with metabolic thermogenesis involving intra- and inter-tissue events and with brown adipose tissue (BAT) thermogenesis (if appropriate).

Modified from Landsberg (1986) and Clark *et al.* (1991).

1991), and premature aging of the blood vessel wall augments the response to agents such as 5-HT (reviewed by Vanhoutte & Cohen, 1983). There appears to be conflicting evidence for a direct role of 5-HT in hypertension (reviewed by van Zwieten *et al.*, 1992), although vascular responsiveness to agents including 5-HT (and NE) are often augmented in diseased animals (reviewed by van Nueten & Janssens, 1988), including the hindlimb of spontaneously hypertensive rats (Cheng & Shibata, 1980).

Atherosclerosis is characterized by intimal proliferation, although lesions are displaced outwards (remodelling) until compensatory mechanisms fail and encroachment on the lumen begins. Impairment of EDRF release, and potentiation of vasoconstrictor responses to 5-HT are associated with this disease (reviewed by Heistad *et al.*, 1991).

1.2 Measurement of skeletal muscle metabolism.

Since early this century, hindquarters of various large animal models have been used to study the control mechanisms of metabolism in skeletal muscle (reviewed by Ruderman *et al.*, 1971; Stainsby & Lambert, 1979). Around 1960, experiments on the hindquarter of the rat were reported by various investigators (reviewed by Ruderman *et al.*, 1971). The calorigenic action of NE was first demonstrated in the leg muscles of dogs by Issekutz *et al.* (1950). Since then, perfused hindlimbs have been utilized to demonstrate the effects of, amongst other things, insulin and α– and β–adrenoceptor stimulation on $\dot{V}O_2$, glucose uptake, lactate release and exercise performance (Ruderman *et al.*, 1971, 1977; Grubb & Folk, 1976, 1977; Chiasson *et al.*, 1981; Richter *et al.*, 1982a; Côté *et al.*, 1985; Schadewaldt *et al.*, 1985; Colquhoun *et al.* 1988, 1990; Ye *et al.*, 1990b; Hettiarachchi *et al.*, 1992). The results from normal, warm-acclimated rats have been compared to hypo- or hyper-thyroid, cold-acclimated, exercise-trained, diabetic and hypertensive rats.

The effects of agents, including insulin and adrenergic agonists, on metabolism in isolated incubated skeletal muscles have also been determined (Challiss *et al.*, 1986; Wallberg-Henriksson *et al.*, 1988; Dimitriadis *et al.*, 1991; Hettiarachchi *et al.*, 1992). However, it was shown that metabolic rate did not increase in incubated muscles upon addition of catecholamines (Hannon *et al.*, 1963; Eaton & Vaughan, 1964), suggesting the importance of a functional vascular system when assessing the

metabolic actions of vasoconstrictors.

1.2.1 Composition of rat hindlimb.

In the rat, the hindquarter contributes almost 20% of body mass (Ruderman *et al.*, 1971). Thus alterations in skeletal muscle blood flow and metabolism have the potential to affect whole body maintenance of blood pressure and metabolism. The hindlimb of 170-230 g rats is predominantly composed of skeletal muscle (77%) with minor components including bone (10%) and skin (12%), and the mass of muscle is proportional to the total body weight of the rat (8.3%) (Ruderman *et al.*, 1971).

1.2.1.1 Skeletal muscle fibres.

Skeletal muscle fibres have different oxidative capacity, thus changes in blood flow may alter the nutrient usage of the muscles. Of the rat hindlimb muscle fibres, less than 10% are slow-twitch oxidative, approximately 40% are fast-twitch oxidative/glycolytic, and the remainder are fast-twitch glycolytic (Laughlin & Armstrong, 1983). It is generally, but not always (Maxwell *et al.*, 1980), accepted that capillary and mitochondrial density decrease in parallel with oxidative capacity, thereby decreasing the diffusion distance for delivered oxygen (Gauthier, 1971; Eriksson & Myrhage, 1972). Although fast-twitch glycolytic and slow-twitch oxidative fibres tend to have similar glycogen stores (Richter & Galbo, 1986), fast-twitch glycolytic fibres fatigue rapidly (Segal *et al.*, 1986), and glycogen stores are markedly depleted during contractions (Richter & Galbo, 1986), suggesting a dependence on anaerobic metabolism.

1.2.1.1.1 Mechanism of skeletal muscle contraction.

Skeletal muscle fibres are innervated by large myelinated nerve fibres. Action potentials spread towards the end of each muscle fibre, allowing coordinated contraction, and signal the release of Ca^{2+} from the sarcoplasmic reticulum (reviewed by Guyton, 1986), possibly via the generation of inositol triphosphate (IP_3, reviewed by Hidalgo & Jaimovich, 1989). Simplistically, muscle contraction is based on the interaction of two proteins, myosin and actin, in the presence of ATP. Actomyosin has a high Mg^{2+}-ATPase activity that is inhibited by troponin. Ca^{2+} removes the

inhibitory effect of troponin (Ebashi, 1971). ATPase activity causes the ADP levels to rise which in turn is the signal to intermediary metabolism for the observed increased through-put of substrates for the generation of ATP. ATP supplies the energy to actuate the "walk-along" mechanism by which the cross-bridges pull the actin filaments, for Ca^{2+} pumping from the sarcoplasm into the sarcoplasmic reticulum, and for pumping Na^{+} and K^{+} through the muscle fibre membrane to maintain an appropriate ionic environment for the propagation of action potentials.

1.2.1.1.2 Energetics of skeletal muscle contraction.

Skeletal muscle phosphocreatine stores can supply energy for the first few seconds of contraction, but for maintained contraction either aerobic or anaerobic generation of ATP is required. Glycolytic ATP formation is approx. 2.5 times more rapid than by oxidative metabolism, but generally supplies less energy in total (Guyton, 1986). Insulin (James *et al.*, 1988) and exercise (Douen *et al.*, 1990) have both been shown to translocate the unique GLUT 4 glucose transporters to skeletal myocyte cell membranes from distinct internal stores (Douen *et al.*, 1990; Marette *et al.*, 1992) by an as yet not fully determined mechanism. Elevation of cAMP (eg. with β-adrenergic agonists) activates triglyceride lipase and, subsequently, lipolysis. Exercise-stimulated fatty acid oxidation by an unknown mechanism generates the main source of ATP during contraction.

1.2.1.2 Vascular System.

The vascular bed in skeletal muscle is considered to be haemodynamically one of the most important circuits in the systemic circulation (Lindbom & Arfors, 1984). The function of vascular resistance in this tissue is fundamental for the blood pressure homeostasis. Local processes within the muscle tissue adjust the flow and exchange according to tissue metabolic needs by coordinating the responses of resistance and exchange vessels in the muscle (reviewed by Bevan, 1987, and Mulvany & Aalkjær, 1990).

Direct intravital observation of the vascular bed of resting skeletal muscle *in situ* demonstrated that small arterioles generally exhibit pronounced vasomotion (Tangelder *et al.*, 1984). It was proposed that these oscillatory changes in blood flow were not associated with sympathetic nerve activity, but an interaction between K^{+}-

channels and potential-dependent Ca^{2+}-channels (reviewed by Mulvany & Aalkjær, 1990). Vasomotion activity has been shown to be greatest at the terminal arterioles (Meyer *et al.*, 1987) and decreased upon addition of vasodilators or reduction of arterial pressure in muscle due to aortic occlusion (Meyer *et al.*, 1988; Slaaf *et al.*, 1989). However, in the latter studies oxygen delivery to muscle may have been markedly reduced.

It has been suggested that in situations of decreased flow velocity or elevated metabolic rate, smooth muscle partial pressure of oxygen (PO_2) decreases resulting in vasodilatation and an increase in blood flow (Duling & Berne, 1970; reviewed by Duling & Klitzman, 1980). Despite earlier suggestions, it has recently been found that oxygen did not have a direct effect on arterioles (Boegehold & Johnson, 1988). Jackson (1987) suggested oxygen reactivity was mediated by either a tissue sensor or by a sensor within the distal portions of the microvasculature. This idea has gained support due to ability of terminal arterioles and capillaries to conduct responses along the arterial network to the proximal arterioles that control the distribution and magnitude of flow by either vasodilatation or constriction (Jackson, 1987; Segal, 1991). The mechanism of this cell-to-cell conduction is currently being investigated, with recent evidence highlighting the importance of smooth muscle and endothelial cell coupling via gap junctions (Segal & Bény, 1992).

Peripheral vasomotor tone is a complex interrelationship between neural and hormonal vasoactive compounds. Thus in situations where tone begins to compromise tissue function, the local metabolic vasodilatory processes can override the tone. Conversely, it is also possible that tone regulates the flow to the tissue and may prevent unnecessary usage of fuels, for example, in the resting state flow through brown adipose tissue of rats is low due to increased tone on the arteries supplying the abundant capillary plexus (Nnodim & Lever, 1988). However in the absence of futile cycles or uncoupling, it must be argued that increases in delivery of fuels to uncompromised resting tissue would not increase metabolic rate.

1.2.1.2.1 Mechanism of vascular smooth muscle contraction.

The mechanism of vascular smooth muscle contraction is similar to that of skeletal muscle in that activation of the myosin ATPase, and thus contraction, is dependent on increases in cytosolic Ca^{2+} (reviewed by Somlyo & Somlyo, 1992). However, myosin light chain phosphorylation and attachment of cross-bridges to actin

does not fully describe cellular processes in smooth muscle contraction. Murphy (1988) proposed a latch state to account for observed maintenance of force with reduced myosin light chain phosphorylation and maximal shortening velocity (reviewed by Stull *et al.*, 1991). Excitation-contraction coupling may be either electromechanical, involving depolarization of the membrane potential by agents including K^+, or pharmacomechanical, involving drug-induced contractions without depolarization (reviewed by Somlyo & Somlyo, 1992). Electromechanical coupling may use both Ca^{2+} influx and release from the sarcoplasmic reticulum, and in general is more sensitive to Ca^{2+} entry blockers or removal of extracellular Ca^{2+} (Somlyo & Somlyo, 1992). Pharmacomechanical coupling involves ligand-gated Ca^{2+} influx, intracellular Ca^{2+} release by IP_3, and modulation of the Ca^{2+} sensitivity of the contractile regulatory apparatus; and is usually accompanied by electromechanical coupling (reviewed by Somlyo & Somlyo, 1992).

1.2.1.2.2 Energetics of vascular smooth muscle contraction.

Energy for contraction of smooth muscle is predominantly generated by mitochondrial oxidative phosphorylation, and is compartmentalized, at least in some vessels, from glycolytically derived ATP utilized for membrane Na^+ transport (Lynch & Paul, 1983). In incubated rabbit aorta rings, fatty acid oxidation accounted for almost all the basal $\dot{V}O_2$, and a large proportion of the fatty acids were derived from endogenous tissue stores (Odessey & Chace, 1982). There appears to be a marked heterogeneity in fuel-dependence during smooth muscle contraction, which appears to be related to both blood vessel size and anatomical location (discussed in detail in Chapter 3).

The amount of phosphagen in vascular smooth muscle is small in comparison to skeletal muscle (Paul, 1977), and there appears to be a large proportion of UTP (Clark *et al.*, 1990). This suggests purine (and pyrimidine) triphosphate utilization and its metabolic resynthesis is tightly coupled, particularly in a temporal sense (Paul, 1977). During vasoconstriction, but not skeletal muscle contraction, large increases in the release of purines and pyrimidines have been observed (Clark *et al.*, 1990; Richards *et al.*, 1992, 1993, submitted 1993). The breakdown products of ATP and UTP appear to be released from working tissue and subsequently rapidly degraded by endothelial ectonucleotidases to uric acid and uracil (Richards *et al.*, 1993). Thus it appears that in addition to ATP, UTP may be a major fuel utilized during

vasoconstriction (Daemers-Lambert, 1964; Clark *et al.*, 1990; Richards *et al.*, submitted 1993).

1.2.1.2.3 Mechanism of vascular smooth muscle dilatation.

Hyperpolarization of the surface membrane of smooth muscle causes relaxation by reducing cytoplasmic Ca^{2+} by closing voltage-gated Ca^{2+} channels and by promoting Ca^{2+} uptake into the sarcoplasmic reticulum (Clapp & Gurney, 1991). The inhibitory action of drugs can also include pharmacomechanical components that are not dependent on changes in the surface membrane potential (Somlyo & Somlyo, 1992). For example, vasodilatation via stimulation of β–adrenoceptors occurs primarily due to activation of adenylate cyclase, thereby forming cAMP, which can reduce intracellular [Ca^{2+}] by decreasing Ca^{2+}-release from the sarcoplasmic reticulum (McDaniel *et al.*, 1991). In addition to a reduction in intracellular [Ca^{2+}], stimulation of guanylate cyclase and formation of cGMP by agents such as nitrovasodilators also separate the generation of tension from myosin phosphorylation by an unknown mechanism (McDaniel *et al.*, 1992).

1.2.2 Resting rat hindlimb as a model of skeletal muscle.

1.2.2.1 In situ studies.

Jansky and Hart (1963) were the first to evaluate rat hindquarter $\dot{V}O_2$. These authors showed that cold-acclimating rats and infusion of NE increased basal $\dot{V}O_2$. The response to NE occurred in the absence of a change in flow to the muscle, and was attributed to an increased $\dot{V}O_2$ by skeletal muscle (Jansky & Hart, 1963). These authors evaluated the extent of muscle perfused in the rat hindquarter by injecting India ink via the femoral artery and fixing the leg in formaldehyde. Using a visual scale of 0-4, 4 indicating total blackening, it was found that 63% of muscle were 4 and most of the remaining muscle were between 2 and 3 (Jansky & Hart, 1963). Unfortunately, the authors did not repeat the procedure in the presence of NE.

A technique often utilized is the autoperfusion of hindlimbs (usually with constant flow) with blood from the carotid artery pumped into the femoral artery. In this procedure, circulating vasoactive agents are not removed, yet nicotinic and muscarinic acetylcholine receptors and β–ARs are usually blocked. In autoperfused

preparations it is generally considered that NE stimulates α_1- and α_2-ARs (Timmermans & Van Zwieten, 1980; Medgett & Ruffolo, 1987; Faber, 1988; Nielsen *et al.*, 1990). Adrenergic constriction of small, precapillary arterioles was due to stimulation of α_2-ARs, whereas constriction of large arterioles and venules was due to stimulation of α_1- and α_2-ARs (Faber 1988, Ohyanagi *et al.*, 1991). Interestingly, vasoconstriction mediated by stimulation of α_2-ARs, and to a lesser extent α_1-ARs, was inhibited by endothelium-derived relaxing factor (EDRF) (Ohyanagi *et al.*, 1992). Earlier findings had proposed endothelial α_2-ARs directly stimulated EDRF release (Angus *et al.*, 1986).

1.2.2.2 Isolated hindlimb perfused with constant pressure.

Few studies have perfused isolated rat hindlimbs with constant pressure. In one study, hindlimbs were perfused at 20°C with a modified Tyrode solution containing 5% bovine serum albumin (van Meel *et al.*, 1983). Stimulation of both α_1- and α_2-ARs decreased flow, and stimulation of α_2-ARs, but not α_1-ARs, was sensitive to Ca^{2+}-entry blockers (van Meel *et al.*, 1983). No effects on $\dot{V}O_2$ were measured.

1.2.2.3 Isolated hindlimb perfused with constant flow.

Isolated rat hindquarter preparations have been perfused with various media, rat sizes, flow rates and temperatures. Variables in media include the presence of erythrocytes and/or albumin.

1.2.2.3.1 Erythrocyte-perfused.

Experiments using erythrocyte-perfused hindlimbs were usually performed at 37°C. Erythrocytes from varying species, including human (Ruderman *et al.*, 1971, 1980), porcine (Grubb & Folk, 1976, 1977), and bovine (Karlsson *et al.*, 1975; Lewis *et al.*, 1977), have been washed and added to buffered (usually modified Krebs-Henseleit) perfusion medium. Most perfusion mediums also contained either serum albumin or dextran (usually 4%).

Ruderman *et al.* (1971) made a systematic effort to compare the activities of the isolated rat hindquarter (hindlimb) preparation with the hindquarter *in situ*. These

workers perfused rat hindquarters at 37°C with medium containing Krebs-Henseleit buffer, 4% bovine serum albumin and aged washed human erythrocytes. They compared muscle oxygen, glucose and ketone body uptake, lactate and K^+ release, and the tissue concentrations of creatine phosphate and the adenine nucleotides (Table 1.1). In addition, muscle appearance under the electron microscope was determined and the responses to infusion of insulin and electrical stimulation of the hindquarter were compared between *in situ* and *in vivo* preparations. The results suggested the behaviour of the perfused hindquarter closely resembled that of the hindquarter of the intact rat. Other studies by Ruderman and co-workers in later years (Ruderman *et al.*, 1977; Ruderman & Berger, 1974; Ruderman & Goodman, 1973, 1974) provided further evidence to support their earlier findings (Ruderman *et al.*, 1971) suggesting the isolated perfused rat hindquarter preparation was suitable for metabolic studies.

1.2.2.3.2 Erythrocyte-free.

In the absence of added erythrocytes, various media have been employed to perfuse rat hindlimbs. The viability of perfusing with a modified Krebs-Henseleit buffer in the presence of 4% BSA at 37°C (Strohfeldt *et al.*, 1974), 2% BSA at 25°C (Colquhoun *et al.*, 1990) or absence of albumin at 32°C (Shiota & Sugano, 1986) was assessed by comparing tissue concentrations of high energy phosphates and glycogen in perfused and unperfused muscle (Tables 1.1 & 1.2). It was found that omitting erythrocytes, or decreasing [BSA] and temperature had no significant effect on tissue metabolites in comparison to levels observed *in vivo* or using erythrocyte-perfused hindlimbs.

The vasoconstrictor effects of hormones have also been determined by perfusing hindlimbs with Krebs-Ringer medium containing 4% dextran (Cheng & Shibata, 1980), and modified Tyrode solution also containing 4% dextran (Verheyen *et al.*, 1991), or 3% artificial colloid (Ficoll) (Åhlund *et al.*, 1977).

1.3 Effects of norepinephrine in constant-flow perfused hindlimb.

NE is thought to stimulate α– and β–adrenoceptors (AR) in rat hindlimb (Grubb & Folk, 1977; Richter *et al.*, 1982a; Côté *et al.*, 1985; Colquhoun *et al.*, 1990; Hettiarachchi *et al.*, 1992). Stimulation of β–ARs is generally associated with vasodilatation (Colquhoun *et al.*, 1990; Hettiarachchi *et al.*, 1992) and increased

Medium	Wet wt / Dry wt	Metabolite (μmol.g wet wt^{-1})				Ref.
		ATP	ADP	CrP	Glycogen	
In Vivo						
	N.D.	5.0 ± 0.2	0.75 ± 0.01	15.6 ± 0.8	N.D.	A
	N.D.	6.4 ± 0.2	N.D.	18.5 ± 0.6	33.8 ± 2.0	B
	4.4 ± 0.1	6.2 ± 0.2	0.80 ± 0.05	16.4 ± 0.9	27.4 ± 1.3	C
	*	4.1 ± 0.0	0.73 ± 0.03	16.6 ± 1.7	N.D.	D
Perfused						
(I)	N.D.	5.2 ± 0.3	0.95 ± 0.09	15.1 ± 0.6	N.D.	A
(II)	N.D.	7.9 ± 0.4	N.D.	20.1 ± 1.5	36.0 ± 2.9	B
(IIIa)	4.7 ± 0.1	5.4 ± 0.1	0.62 ± 0.04	14.6 ± 0.2	30.8 ± 2.6	C
(IIIb)	5.5 ± 0.3	4.9 ± 0.2	0.69 ± 0.09	12.2 ± 0.2	22.5 ± 1.4	C
(IIIc)	6.1 ± 0.1	3.9 ± 0.3	0.38 ± 0.03	11.0 ± 0.3	25.9 ± 0.6	C
(IV)	5.8	3.1 ± 0.2	0.45 ± 0.03	14.1 ± 0.8	N.D.	D

Table 1.1 Metabolite concentrations in rat hindlimb skeletal muscle.

Hindlimbs from anaesthetized rats of varying size were either freeze-clamped (*in vivo*) or perfused at a constant flow-rate with modified Krebs-Henseleit perfusion medium gassed with 95% O_2-5% CO_2 and containing either 4% bovine serum albumin (BSA) with (I) or without (II) erythrocytes (RBC) at 37°C, 2% BSA without erythrocytes at 25°C (III), or without erythrocytes or BSA at 32°C (IV): A, from Ruderman *et al.* (1971); B, from Strohfeldt *et al.* (1974); C, from Colquhoun *et al.* (1990) after 50 min perfusion (a) or 180 min perfusion in the absence (b) or presence (c) of vasopressin; D, from Shiota and Sugano (1986); *, assuming wet wt/dry wt = 4.4 (C). N.D., not determined. Values are means ± SE.

Ref.	Temp (°C)	Flow Rate (ml.min^{-1}.g^{-1})	Oxygen Uptake	Lactate Release	Pyruvate Release	Glucose Uptake	Glycerol Release
			(μmol.h^{-1}.g^{-1})				
A	37	0.30(+)	21.6 ± 1.6	5.4 ± 1.2	N.D.	1.0 ± 0.6	0.5 ± 0.04
B	37	1.31(-)	30.6 ± 0.4	7.0 ± 0.9	N.D.	7.8 ± 0.4	0.3 ± 0.02
C	25	0.27	6.4 ± 0.2	6.4 ± 4.8	0.6 ± 0.2	N.D.	N.D.
D	32	0.88	10.5	21.1	3.5	2.1	N.D.

Table 1.2 Rates of metabolism in hindlimb skeletal muscle.

Hindlimbs from rats of varying size were perfused at a constant flow-rate with modified Krebs-Henseleit perfusion medium gassed with 95% O_2-5% CO_2 and containing either 4% bovine serum albumin (BSA) with (+) or without (-) erythrocytes at 37°C, 2% BSA without erythrocytes at 25°C, or without BSA or erythrocytes at 32°C: A, from Ruderman *et al.* (1971); B, from Strohfeldt *et al.* (1974); C, from Colquhoun *et al.* (1990); D, from Shiota and Sugano (1986); N.D., not determined. Values are means, and when available, ± SE.

glycogenolysis (Richter *et al.*, 1982a), whereas α–AR stimulation leads to vasoconstriction (Grubb & Folk, 1977; Côtè *et al.*, 1985; Colquhoun *et al.*, 1990; Hettiarachchi *et al.*, 1992). In erythrocyte-free, constant-flow preparations the vasoconstrictor response to NE was blocked by the α_1-AR antagonist prazosin (Grubb & Folk, 1977), thereby leaving in doubt the occurrence of α_2-AR stimulation. The apparent absence of an α_2-AR response may be due to a lack of sufficient basal tone using *in vitro* perfusion techniques (McGrath *et al.*, 1990).

1.3.1 Sites of NE-induced effects.

Subtypes of α–ARs have been shown to be located on both arterial and venous smooth muscle within the microcirculation of skeletal muscle (Faber, 1988). Results from various reports suggest stimulation by lower doses of NE caused vasoconstriction of smaller arterioles. Direct observation (Gray, 1971), analysis of the number of adrenergic nerve terminals and receptors surrounding different sized vessels (Hirst & Edwards, 1989), and dependence on extracellular Ca^{2+} during vasoconstriction (Sutter *et al.*, 1977), all support the constriction of small arterioles by low doses of NE in skeletal muscle. Similarly, β–ARs also appear to be located on smaller, rather than larger vessels (Lundvall & Hillman, 1978; Lundvall *et al.*, 1982). The increased responsiveness to NE of arterioles within fast-twitch glycolytic muscle (Gray, 1971) suggests flow may increase to slow-oxidative muscle upon infusion of low doses of NE.

In contrast, higher doses of NE begin to stimulate α_1-ARs located on larger vessels (Gray, 1971; Faber, 1988) which are capable of extracellular Ca^{2+}-independent vasoconstriction (Sutter *et al.*, 1977).

1.3.2 Thermogenic effects of NE.

Since the work of Mejsnar and Jansky (1971) and Ruderman *et al.* (1971) the constant-flow perfused rat hindlimb has been used as a model to investigate NE-induced changes in $\dot{V}O_2$. In this system, NE has repeatedly been demonstrated to dose-dependently increase basal $\dot{V}O_2$ by a maximum of 50-100% in associated with increases in perfusion pressure during constant-flow perfusion using physiological medium with red blood cells at 37°C (Table 1.3; Mejsnar & Jansky, 1971; Grubb &

Ref.	Flow Rate ($ml.min^{-1}.g^{-1}$)	[NE] (nM)	Oxygen Uptake ($\mu mol.h^{-1}.g^{-1}$)		Perfusion Pressure (mmHg)	
			Basal	Change	Basal	Change
A	0.08	300 3000	17.3 ± 1.8	15 -2	25	15 90
B	0.48	*	24.0 ± 1.0	13	59.2 ± 2.0	40
C	0.21	10 63	24.6 ± 1.5	4 8	43.5 ± 1.7	5 20
D	0.27	200 2000	12.0 ± 0.4	7 -2	50	60 100
E	0.27	50	6.4 ± 0.2	3.1 ± 0.1	29.0 ± 1.0	15
F	1.03	50	13	0.2	90	80

Table 1.3 Effect of NE on $\dot{V}O_2$ and perfusion pressure in constant-flow perfused rat hindlimb.

Hindlimbs from rats of varying size were perfused at a constant flow-rate with modified Krebs-Henseleit perfusion medium gassed with 95% O_2-5% CO_2 and containing either 4% bovine serum albumin (BSA) and erythrocytes (RBC) at 37°C (A, B), 2% BSA without erythrocytes at 25°C (C-E), or without BSA and erythrocytes (F): A, from Grubb and Folk (1976), maximum and minimum effects on $\dot{V}O_2$; B, from Richter *et al.* (1982a), *, 24 nM epinephrine + 10 μM (+)-propranolol; C, from Hettiarachchi, 1992; D, from Côté *et al.* (1985), maximum and minimum effects on $\dot{V}O_2$; E, from Colquhoun *et al.* (1988), maximal effect on $\dot{V}O_2$; F, from Shiota and Masumi (1988); $\dot{V}O_2$, oxygen uptake. Values are means, and when available, ± SE.

Folk, 1976; Richter *et al.*, 1982a), or without red blood cells at 25°C (Côté *et al.*, 1985).[2]

Work at the Department of Biochemistry, University of Tasmania had found that vasoconstrictors such as vasopressin and angiotensin II as well as NE increased hindlimb $\dot{V}O_2$ and lactate release in association with increases in perfusion pressure in a dose dependent manner (Colquhoun *et al.*, 1988; Hettiarachchi *et al.*, 1992). These effects occurred over physiological dose ranges and were rapidly reversible upon agonist removal. The explanation for vasoconstrictor-induced thermogenesis is not fully elucidated, although a series of proposals has been developed by either analogy to, or direct evidence from, the literature (reviewed by Colquhoun & Clark, 1991).

1.3.2.1 Skeletal muscle thermogenesis.

It was initially considered that NE directly stimulated skeletal muscle thermogenesis. It is widely accepted that stimulation of β–adrenoceptors (β–AR) led to increased muscle glycogenolysis (Richter *et al.*, 1982a) which was observed in both perfused and incubated muscle preparations (Hettiarachchi *et al.*, 1992). However, in the presence of propranolol, NE-induced $\dot{V}O_2$ was not inhibited suggesting an α–AR mechanism (Colquhoun *et al.*, 1990). However, the small populations of α_1-AR detected in skeletal muscle preparations were thought to be due to vascular smooth muscle α–AR contaminants (Martin *et al.*, 1990). This evidence in conjunction with the inhibition of NE-induced but not contraction-induced thermogenic effects by vasodilators, including nitroprusside, and the additivity of vasoconstrictor- and contraction-induced increased $\dot{V}O_2$ led to the proposal that NE did not directly stimulate skeletal muscle metabolism (Colquhoun *et al.*, 1988, 1990; Hettiarachchi *et al.*, 1992). In addition, perifused skeletal muscle preparations were found to have

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2. However, in experiments where BSA was omitted from the medium, the hindlimb response to NE (50 nM) in warm-, but not cold-, acclimated rats on $\dot{V}O_2$ was almost totally absent despite rises in perfusion pressure (Table 1.3; Shiota & Masumi, 1988). When work of Shiota and Masumi (1988) was duplicated in this laboratory, despite gross oedema, the $\dot{V}O_2$ and perfusion pressure responses to NE (50 nM) were observed, and found to be consistent with observations with albumin added to the perfusion medium (Hettiarachchi, 1992).

only minor thermogenic responses to NE (Dubois-Ferrière & Chinet, 1981; Hettiarachchi *et al.*, 1992) and angiotensin II (Hettiarachchi *et al.*, 1992), although a β-adrenergic response to increase lactate release was observed suggesting adequate diffusion of hormones (Hettiarachchi *et al.*, 1992).

1.3.2.2 Heterogeneity of flow.

It has been consistently observed, but not always reported, that infusion of doses of NE which increased $\dot{V}O_2$ led to the washout of a small number of erythrocytes (Clark *et al.* 1990), less than 10% of the total cleared from the hindlimb. There may be various explanations for this washout, including the perfusion of previously unperfused or underperfused regions. The washout was observed when one or two hindlimbs were perfused, and the presence of torso, tail and abdominal tourniquets suggested a heterogeneity of microvascular flow at the level of the capillaries rather than flow to other tissues (unpublished observation).

The possibility that addition of vasoconstrictors controls access of the perfusate to large areas of skeletal muscle (an additional 65% based on the NE-mediated increase in $\dot{V}O_2$) capable of increased metabolism would require that the hindlimb was partially hypoxic in the basal perfused state. Hypoxia has detrimental effects on the adenine nucleotide pool in perfused skeletal muscle, but no changes in adenine nucleotides, phosphocreatine, glucose-6-phosphate, glycogen, or lactate were observed after 3 h perfusion in the absence or presence of vasopressin (Table 1.1, Colquhoun *et al.*, 1990). In addition, only small quantities of hypoxanthine were produced by this system (Clark *et al.*, 1990). The response to vasoconstrictors on $\dot{V}O_2$, perfusion pressure and release of purine and pyrimidine products was sustained and quantitatively reproducible independently of the period between infusions (Richards *et al.*, 1992, submitted 1993). This observation was in direct contrast to the washout of purines and pyrimidines upon reperfusion of ischaemic regions, where release levels were proportional to the time of stopped flow (Richards *et al.*, submitted 1993).

Other evidence refuting the presence of hypoxic or ischaemic regions comes from perfusions performed at higher flow rates. When flow rates were increased by more than 4-fold, thereby reducing the possibility of hypoxic regions, infusion of NE was still observed to stimulate marked increases $\dot{V}O_2$ and pressure. In addition,

infusion of the vasodilator nitroprusside decreased basal $\dot{V}O_2$ from control (Ye *et al.*, 1990b).

1.3.2.3 Heterogeneity of flow to highly metabolically active regions.

Another possibility for increased $\dot{V}O_2$ and lactate release with vasoconstrictor agonists is that the vasoconstrictors increase the rate of one or more forms of futile cycling or ion pumping in the hindlimb, with the consequent increases in ATP hydrolysis leading to increased metabolism. This would necessitate either receptors for the agonists directly coupled to these events, or the specific redirection of flow to otherwise quiescent regions which were substrate limited and/or lacked a signal. This latter possibility is consistent with the findings of both Honig *et al.* (1982) where functional capillary density in resting skeletal muscle was both actively and passively controlled by arterioles, and the classical work by Verzár (1912) and more recently Chinet and Mejsnar (1989) where flow, and thereby oxygen availability, was related to $\dot{V}O_2$ by cells. It must be noted that decreasing oxygen delivery to skeletal muscle did not always affect $\dot{V}O_2$ (Stainsby & Otis, 1964). However, if the relationship does hold, either oxygen of an alteration in ATP demand may regulate muscle metabolism, a so called "metabolic switch" (reviewed by Arthur *et al.*, 1992).

In the insect, flight muscle substrate cycling is suppressed during contractions (Clark *et al.*, 1973), thus by analogy, substrate cycling could be expected to be inhibited during skeletal muscle contraction. This does not appear so, as contraction- and angiotensin II-induced $\dot{V}O_2$ were additive (Colquhoun *et al.*, 1990). Similar data were found by others with the infusion of epinephrine plus propranolol during contractions in the erythrocyte-perfused hindlimb (Richter *et al.*, 1982a). However in both studies only 30% of the perfused hindlimb mass was contracting (Rennie & Holloszy, 1977).

Alternatively, vasoconstrictors may redistribute flow to specialized heat-producing cells, analogous to the modified eye muscle of the billfish (Block, 1987). In this tissue, Ca^{2+} cycling by the sarcoplasmic reticulum (SR) was proposed to generate heat (Block, 1987). Ca^{2+} cycling has also been implicated in skeletal muscle heat production during malignant hyperthermia (Gronert, 1986) and hyperthyroidism (van Hardeveld & Clausen, 1984). However a direct link between vasoconstriction and futile Ca^{2+} cycling has not been established. There is nevertheless continual pumping of Ca^{2+} from the cytosol into the SR and out of the cell in association with

vasoconstriction, and, if analogous to skeletal muscle, may contribute a large proportion of energy turnover during contraction (reviewed by Clausen *et al.*, 1991; Chinet *et al.*, 1992).

Another possibility (which may not depend on heterogeneous flow) is an endothelial cell shear-stress-dependent control of metabolism. Recent findings in perfused guinea pig hearts have shown that a rise in flow rate increased the shear-stress on the endothelial lining of blood vessels (glycocalyx) thereby stimulating glycolytic flux (Suárez & Rubio, 1991). These authors proposed a from of metabolic coupling between capillary endothelial cells and myocardial cells (Suárez & Rubio, 1991) which may also occur in skeletal muscle.

1.3.2.4 Vascular thermogenesis.

Recent work in this laboratory provided evidence that the vascular system controlled $\dot{V}O_2$ in the perfused rat hindlimb. This evidence also raised the possibility that smooth muscle rather than skeletal muscle metabolism was responsible for the increase in $\dot{V}O_2$ during vasoconstriction, with considerable thermogenesis occurring during constriction and maintenance of tension, giving rise to the notion of vascular thermogenesis or "hot pipes" (Colquhoun *et al.*, 1988, 1990; Ye *et al.*, 1990a, 1990b; Hettiarachchi *et al.*, 1992; reviewed by Colquhoun & Clark, 1991).

This proposal has inherent investigational problems due to difficulties in separating skeletal and smooth muscle metabolism. The argument relies on the assumption that small arterioles have much higher metabolic rates than the well described low rates of metabolism in larger vessels such as the aorta (Paul, 1980). In the light of the apparently small volume of vascular tissue within the hindlimb (approx. 3.4%, Paul, 1980), and Murphy's latch-bridge hypothesis (1988), vasoconstrictors that mediate increases in $\dot{V}O_2$ may stimulate the relatively large number of small arterioles which undergo a form of vasomotion[3]. and thereby stimulate a net large increase in sustained $\dot{V}O_2$. More direct evidence supporting the possibility of vascular thermogenesis comes from agonist-mediated large rises in $\dot{V}O_2$ in association with vasoconstriction in the isolated perfused rat mesenteric artery arcade (Ye *et al.*, 1990a).

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3. Despite the reported decrease in vasomotion with reduced arterial perfusion pressure (Slaaf *et al.*, 1989), the phenomenon of vasomotion has not been investigated in a constant-flow perfusion system that lacks high basal tone yet has adequate oxygen delivery.

1.3.3 Non-thermogenic effects of NE.

Infusion of high concentrations of NE into the hindlimb has been shown to result in dose-dependent decreases of maximum $\dot{V}O_2$ to levels below basal, despite further rises in perfusion pressure (Grubb & Folk, 1976; Côtè *et al.*, 1985). These high concentrations of NE would not be expected to be circulating due to rapid breakdown by monoamine oxidase and catecholamine-*o*-methyl transferase located within vessels. However, high local concentrations of NE may be found within the synaptic cleft and thus it is possible that a major flow-controlling mechanism involves sympathetic vasoconstrictor nerves (Iversen & Nicolaysen, 1990). From perfusion experiments using the isolated dog hindleg and gastrocnemius muscle, Pappenheimer (1941) proposed that reduced $\dot{V}O_2$ was the result of sympathetic nerves acting on blood vessels to divert blood flow from capillaries to arteriovenous anastomoses. Durán and Renkin (1976) investigated this phenomenon in some detail, and although their conclusion differed from Pappenheimer's, they found that high frequency supramaximal stimulation of the lumbar trunk led to an inhibition of $\dot{V}O_2$ with a rise in resistance, but low frequency stimulation increased $\dot{V}O_2$ as well as resistance (Durán & Renkin, 1976). Intermediate frequency stimulation was often associated with an initial fall followed by an increase in $\dot{V}O_2$ and relatively constant resistance (Durán & Renkin, 1976). This pattern of response has been reproduced in this laboratory and was markedly attenuated by α_1-AR antagonists (unpublished observations).

1.4 Effects of serotonin in constant-flow perfused hindlimb.

The vasoconstrictor effects of 5-HT have been reported previously in perfused rat hindlimb (Table 1.4; Åhlund *et al.*, 1977; Cheng & Shibata, 1980; Verheyen *et al.*, 1991), but no associated measurements of $\dot{V}O_2$ were recorded.

Increases in perfusion pressure were totally blocked by ketanserin (5-HT_{2A}-antagonist) or LY 53,857 (general 5-HT_2-antagonist), indicating 5-HT-mediated vasoconstriction occurred via stimulation of 5-HT_2-receptors in the dog hindlimb (Blackshear *et al.*, 1985), rat aorta (Cohen *et al.*, 1983) and to a general extent in most blood vessels including rabbit femoral and tibial arteries (van Nueten, 1985).

Ref.	Temp (°C)	Flow Rate ($ml.min^{-1}.leg^{-1}$)	[5-HT] (μM)	Perfusion Pressure (mmHg)	
				Basal	Δ
A(I)	37	1	1.0	40	60
A(II)	37	1.5	0.0025	26 ± 2	80
B	35	1.0	0.25	15	130
C	38	5.0	1.0	30	200

Table 1.4 Effects of 5-HT on perfusion pressure in constant-flow perfused rat hindlimb.

Hindlimbs from rats of varying size were perfused at a constant flow-rate using perfusion medium gassed with 95% O_2-5% CO_2 containing either dextran (4%): A, from Cheng and Shibata (1980), auto-blood-perfused (I) and Krebs-Ringer-perfused (II); B, from Verheyen *et al.* (1991), Tyrode-perfused; or 3% artificial colloid (Ficoll): C, from Åhlund *et al.* (1977) Tyrode-perfused. Values are means, and when available, ± SE of maximal 5-HT-mediated effects.

1.4.1 Sites of 5-HT-induced effects.

5-HT is generally reported to vasoconstrict larger arteries, and not small arteries or arterioles (Hollenberg, 1985; Blackshear *et al.*, 1985; Tuncer *et al.*, 1992). In skeletal muscle, Harris and co-workers have demonstrated 5-HT-mediated LY 53,857-sensitive constriction of large arterioles and LY 53,857-insensitive dilatation of small arterioles, suggesting the presence of 5-HT_2- and orphan 5-HT-receptors respectively (Wilmoth *et al.*, 1984; Alsip & Harris, 1991).

1.5 The present study.

The importance of the vascular system in the control and contribution to metabolism within skeletal muscle was investigated in the constant-flow perfused rat hindlimb.

The vasoconstrictor and vasodilatory effects mediated by NE and 5-HT were characterized by use of selective receptor antagonists and the marked reduction of oxygen, carbohydrate or Ca^{2+} from the inflow perfusion medium. The agonist-induced effects on tissue metabolism were also investigated by measuring arterio-venous differences in oxygen partial pressure and glucose levels, and the venous concentration of lactate. Final studies investigated agonist-mediated flow heterogeneity.

CHAPTER 2

Vascular effects of NE and 5-HT.

2.1 Introduction.

2.1.1 Vasoconstrictor-mediated increases in skeletal muscle metabolism.

Jansky and Hart (1963) demonstrated that skeletal muscle had an important role in norepinephrine (NE)-induced thermogenesis *in vivo*. Further investigation showed the increases in skeletal muscle oxygen uptake ($\dot{V}O_2$) could be observed and quantified using constant-flow perfusion techniques (Mejsnar and Jansky, 1971). Since then, the constant-flow perfused rat hindlimb has been used as a model to investigate NE-induced oxygen uptake in skeletal muscle. In this system, NE has repeatedly been demonstrated to markedly increase basal oxygen uptake and lactate release during constant perfusion with erythrocytes at 37°C (Grubb and Folk, 1976; Richter *et al.*, 1982a), or without erythrocytes at 25°C (Côté *et al.*, 1985; Colquhoun *et al.*, 1988, 1990; Ye *et al.*, 1990b; Hettiarachchi *et al.*, 1992). These effects were shown to be due to stimulation of α-adrenoceptors (AR) (Grubb and Folk, 1977; Côté *et al.*, 1985; Colquhoun *et al.*, 1990; Hettiarachchi *et al.*, 1992), probably α_1-AR (Grubb and Folk, 1977).

Recent work in this laboratory provided evidence that the vascular system controlled $\dot{V}O_2$ in the perfused rat hindlimb. Vasoconstrictors such as vasopressin and angiotensin II as well as NE increased hindlimb $\dot{V}O_2$ and lactate release in association with perfusion pressure in a dose dependent manner (Colquhoun *et al.*, 1988; Hettiarachchi *et al.*, 1992). These effects were inhibited by the vasodilators nitroprusside, nifedipine and isoproterenol, indicating the involvement of the vascular system (Colquhoun *et al.*, 1988, 1990; Ye *et al.*, 1990b; Hettiarachchi *et al.*, 1992). This argument was strengthened by further work (Ye *et al.*, 1990b; Colquhoun *et al.*, 1990) where (a) flow-induced increases in $\dot{V}O_2$ and pressure were blocked by nitroprusside; (b) at all flow rates NE further increased the effects on $\dot{V}O_2$ and perfusion pressure; and (c) skeletal muscle contraction-induced $\dot{V}O_2$ was additive to $\dot{V}O_2$ uptake produced by vasoconstriction. In addition vasoconstrictors were able to increase $\dot{V}O_2$ and lactate release in a less complex vascular system, the perfused

mesenteric artery arcade (Ye *et al.*, 1990a). The above evidence raised the possibility that smooth muscle metabolism rather than skeletal muscle metabolism was responsible for the increase in metabolism during vasoconstriction, giving rise to the notion of vascular thermogenesis or "hot pipes" (reviewed in Colquhoun and Clark, 1991).

In addition to vasocontrictor effects, NE has both a vasodilatory action in vascular smooth muscle and glycogenolytic action in skeletal muscle, due to stimulation of β-ARs. β-AR stimulation of smooth muscle stimulates cyclic AMP-dependent phosphorylation and enhancement of Na^+/K^+ transport (Scheid *et al.*, 1979). In general, blood vessels contain predominantly β_2-ARs (Fujimoto *et al.*, 1988), although some vessels exhibit β_1-AR-induced vasodilatation (Purdy *et al.*, 1988). The β-AR density appears homogeneous on resistance arterioles within rat hindquarter, independent of surrounding skeletal muscle fibre type (Martin *et al.*, 1989). Colquhoun *et al.* (1990) demonstrated the combination of the β-antagonist propranolol (1 μM) and NE resulted in greater increases in perfusion pressure and $\dot{V}O_2$ than NE alone, for doses of NE greater than 25 nM. Stimulation of β-ARs with isoproterenol (50 nM) in the absence or presence of vasoconstrictors led to decreases in perfusion pressure and $\dot{V}O_2$ (Colquhoun *et al.*, 1990; Hettiarachchi *et al.*, 1992). The β-ARs in skeletal muscle have been shown to increase lactate release due to stimulation of glycogenolysis (Dimitriadis *et al.*, 1991). β_2-ARs appear predominant, but evidence exists for β_1- and β_3-ARs in skeletal muscle (Kim *et al.*, 1991; Molenaar *et al.*, 1991). The receptor density increases with type I, slow-oxidative fibre content and vascular density (Martin *et al.*, 1989; Kim *et al.*, 1991). Consistent with earlier reports (Richter *et al.*, 1982a), isoproterenol stimulated increases in skeletal muscle lactate release in perfused and unperfused muscle (Hettiarachchi *et al.*, 1992).

2.1.2 Vasoconstrictor-mediated decreases in skeletal muscle metabolism.

Infusion of vasoconstrictors into constant-flow perfused hindlimbs did not always lead to increases in metabolism. It was observed that upon infusion of higher doses of NE, perfusion pressure continued to rise, yet $\dot{V}O_2$ began to decrease towards, and even below, basal levels despite constant-flow perfusion (Grubb and Folk, 1976; Côté *et al.*, 1985).

In preliminary experiments in this laboratory, serotonin (5-hydroxytryptamine, 5-HT) was found to stimulate decreases in $\dot{V}O_2$ in association

with rises in perfusion pressure. The reason for this decrease in metabolism was unclear, as there have been no reports of 5-HT directly affecting skeletal muscle metabolism. In general, 5-HT is reported to cause vasoconstriction within skeletal muscle via stimulation of 5-HT_2 (5-HT_{2A} and/or 5-HT_{2C}) receptors on larger arteries, but not on smaller arteries or arterioles (Hollenberg, 1985; Blackshear *et al.*, 1985; Alsip & Harris, 1991). 5-HT-mediated vasodilatation of smaller blood vessels in skeletal muscle has also been unmasked, and was thought to be via stimulation of orphan 5-HT-receptors (Alsip & Harris, 1991).

It was considered of interest to further characterize these apparently anomalous findings with NE and 5-HT in the perfused hindlimb. In the present study, the actions of 5-HT and NE in both the isolated, perfused rat hindlimb and mesenteric arterial arcade were compared. Agonist-mediated effects on perfusion pressure, $\dot{V}O_2$ and lactate release were determined, and the receptors stimulated were assessed by use of selective adrenergic and 5-HT antagonists.

2.2 Materials and methods.

2.2.1 Perfusion techniques.

2.2.1.1 Animal care.

Male hooded Wistar rats were used in all experiments. Five to eight rats were housed per cage under temperature-controlled conditions (12 h:12 h light/dark cycle). Rats were provided with a commercial diet (21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fibre with added vitamins and minerals from Gibson's Hobart) and water *ad libitum*.

2.2.1.2 Surgical procedures.

2.2.1.2.1 Perfused rat hindlimb.

Surgery was performed as described by (Colquhoun *et al.*, 1988), which was a modified version of that used by Ruderman *et al.* (1971). The detailed operative procedure was as follows (and Figure 2.1):

Rats were anaesthetized with an *intraperitoneal* injection of aqueous pentobarbital sodium (6 mg/100 g body weight). After a midline abdominal incision, the skin was reflected and the superior epigastric vessels were ligated (for anatomical nomenclature see Greene, 1968). The abdominal wall was then incised from the pubic symphysis to the xiphoid process. Ligatures were placed around the left superficial epigastric vessel (**1.**), the internal spermatic vessels (**2.**) and other vessels supplying the testes, the neck of the bladder (**3.**) and the seminal vesicles (**4.**). The testes and seminal vesicles were removed. Two ligatures were placed around the descending colon {proximal to the inferior mesenteric artery, (**5.**)} and excised in between. The colon and large intestine were separated from the connective tissue to the level of the renal vessels (**6.**), and a ligature was placed around the duodenum taking care to tie off the superior mesenteric vessels (**7.**). The intestine was excised below the duodenal ligature and the entire gastro-intestinal tract was removed. Ligatures were placed around the iliolumbar vessels (**8.**), ureter (**9.**), tail near the anus (**10.**) and tarsus of the left foot (**11.**). In experiments when only one leg was perfused, flow was restricted to the left limb by ligation of the contralateral common iliac artery (**12.**).

Next, two pairs of loose ligatures were placed around the aorta and the vena cava. The first pair (**13.**) was located immediately below the renal vessels, and the second pair (**14.**) above the iliolumbar vessels. Heparin (1000 units per ml) (0.1 ml/100 g body wt) was injected into the vena cava at the junction between the right renal vein and the vena cava and allowed to circulate. The vena cava (**15.**) was tied off (**13.**), and cannulated by passing a Terumo 18G needle with 16G catheter through the vessel wall (**X**). The catheter tip was positioned 3 mm above the aortic bifurcation and tied in place. The aorta (**16.**) was tied off (**13.**) and a small incision (**X**) allowed insertion of a Terumo 18G catheter filled with 0.9% NaCl. The catheter was pushed gently until the tip was at the same level as the venous catheter, and then secured.

The preparation was then transferred to the perfusion apparatus, and the arterial cannula was connected to the oxygenated perfusion medium inflow line. In the majority of experiments perfusate was pumped into the rat at a constant flow-rate of 4.1 ± 0.1 ml.min^{-1}. The outflow line was connected to the venous cannula (Figure 2.3). Approximately 2 min elapsed from the time the vena cava was ligated and the circulation was re-established. A further ligature was placed around the abdomen of the rat at the level of the L3, L4 vertebrae to restrict flow to the upper torso. The animal was then sacrificed with an over-dose of pentobarbital sodium injected into the

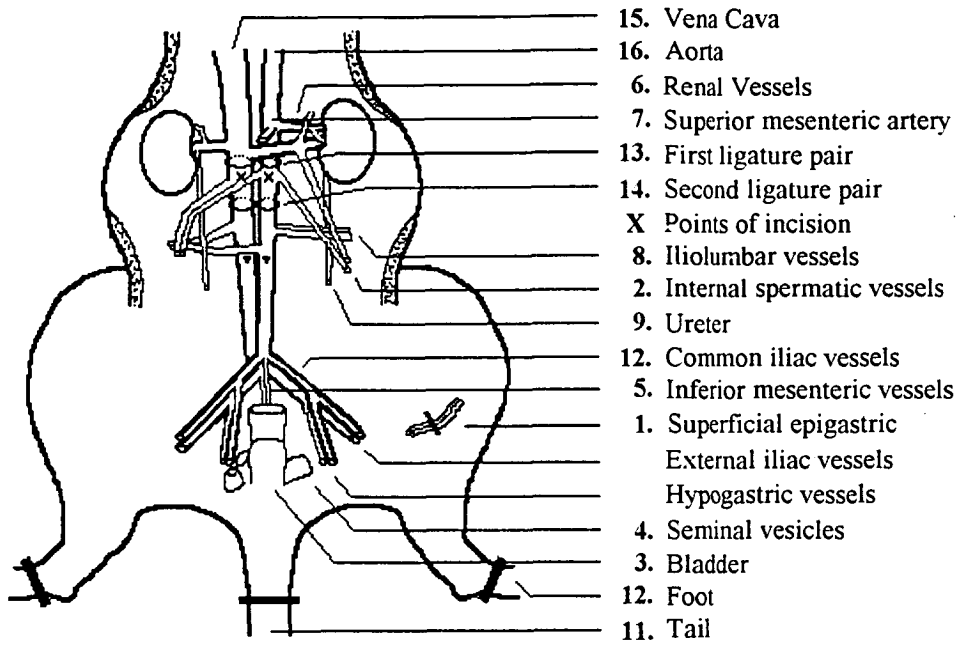

Figure 2.1 Vessels ligated during surgical preparation of the isolated rat hindlimb.

The perfusion method was modified from Ruderman *et al.* (1971) according to Section 2.2.1.2.1. Numbers and names of the vessels are referred to in the text. Modified from Greene (1968) and Ruderman *et al.* (1971).

heart. The entire operative procedure required 20-25 min.

2.2.1.2.2 Perfused rat mesenteric artery arcade.

Perfusion of the superior mesenteric artery arcade was based on that by Ye *et al.*, (1990b) with some major modifications. Animal care, and the perfusion medium and apparatus are described in Sections 2.2.1.1 and 2.2.1.3, respectively. BSA (2%) was added to the modified Krebs-Henseleit bicarbonate buffer, and perfusions were conducted at 25°C. The detailed operative procedure was as follows (Figure 2.2):

Rats (200-250g) were anaesthetized with an *intraperitoneal* injection of aqueous pentobarbital sodium (6 mg/100 g body weight). After a midline abdominal incision, the skin was reflected and the superior epigastric vessels were ligated (for anatomical nomenclature see Greene, 1968). The abdominal wall was then incised from the pubic symphysis to the xiphoid process. Two ligatures were placed around the descending colon (proximal to the inferior mesenteric artery, see Figure 2.2) and excised in between. The colon and large intestine were separated from the connective tissue to the level of the renal vessels.

Next, two loose ligatures were placed around the aorta, the first above the superior mesenteric artery branch-point and second at the level of the left renal vein. A loose ligature was also placed around the superior mesenteric artery 2 mm from aortic branch-point. Heparin (100 U/100 g body wt) was injected into the vena cava below the right iliolumbar vein branch point, and allowed to circulate for 1 min. The first aortic ligature was tied off, and a small incision (**X**) allowed the retrograde insertion of a Terumo 18G catheter (inserted through a rubber stopper) filled with 0.9% NaCl. The catheter was pushed up until the tip was at the superior mesenteric artery branch-point then was gently manoeuvred approx. 4 mm into this vessel. The lower aortic and mesenteric ligatures were then secured.

The arterial cannula was connected to the oxygenated perfusion medium inflow line, and flow commenced through the arcade at 1.5 $ml.min^{-1}$. Approximately 1 min elapsed from the time the aorta was ligated and the circulation was re-established. The intestine was then carefully cut away from the arcade microcirculation, thereby preventing capillary flow and venous return. The artery arcade was removed from the rat, bathed in perfusion medium to remove any residual red blood cells, and then transferred into the perfusion chamber. The chamber consisted of a glass tube tapered at the end (ID 16 mm, total volume 6 ml). The

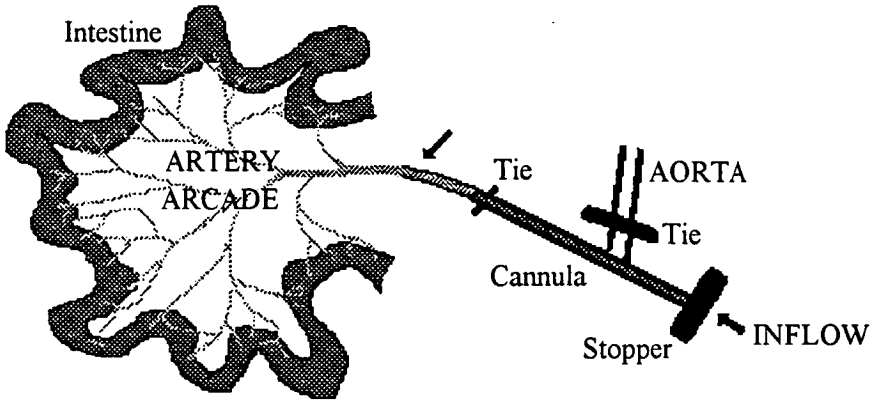

Figure 2.2 Vessels ligated during the surgical preparation of the isolated rat mesenteric artery arcade.

The perfusion method and surgical preparation was modified from Ye *et al.* (1990a) according to Section 2.2.1.2.2. The cannulated arterial arcade was placed in a fluid-filled chamber (inset) to prevent oxygen loss to the atmosphere.

stopper on the cannula sealed the chamber, allowing upward perfusion, and filling of the chamber with perfusion medium. The outflow line was connected to the tapered end of the chamber. The animal was then sacrificed with an over-dose of pentobarbital sodium. The entire operative procedure required 20-25 min. 0.2 ml of Evans Blue dye was infused proximal to the arterial cannula to determine whether the entire network was being perfused before commencement of experiments, and on completion of experiments to allow determination of perfused mass. Unperfused tissue(predominantly lymph and fat deposits) was carefully dissected away, the preparation was then blotted and wet weight measured.

2.2.1.3 Perfusion medium.

The standard perfusion medium was prepared according to Côté *et al.* (1985). 2% (w/v) bovine serum albumin (BSA) was added to Krebs-Henseleit bicarbonate buffer (118 m NaCl, 4.74 mM KCl, 1.19 mM KH_2PO_4, 1.18 mM $MgSO_4$, 25 mM $NaHCO_3$) including 1.27 mM $CaCl_2$ and 8.3 mM glucose. BSA was dialysed 5 times against distilled water before spectophotometric (540 nm) determination of protein content (Biuret Assay, Clark, 1964). The final solution was filtered (0.45 μm) before use.

In experiments performed at higher temperatures, 4% BSA was added to the Krebs-Henseleit medium (Chapter 5) in addition to fresh, washed bovine erythrocytes (Chapter 6).

2.2.1.4 Perfusion apparatus.

Perfusions were conducted in a thermostatically-controlled chamber (Figures 2.3 & 2.4). Unless specified otherwise, the perfusion temperature was 25°C. This reduced basal oxygen uptake by hindlimb and mesenteric artery tissues and improved O_2-solubility, thereby allowing adequate O_2 delivery by a non-erythrocyte medium as described by Côté *et al.* (1985). The perfusion medium reservoir was gassed with 95% O_2-5% CO_2 maintained at 4°C to enable full oxygenation. Gassed perfusate was pumped at a constant-flow rate by a peristaltic pump (Masterflex, Cole Palmer, USA) from the reservoir through the apparatus. Before reaching the hindlimb (or mesenteric artery arcade), the medium passed through a water-jacketed glass heat exchange coil, and an oxygenator. The oxygenator consisted of a glass jar containing 3 meters of

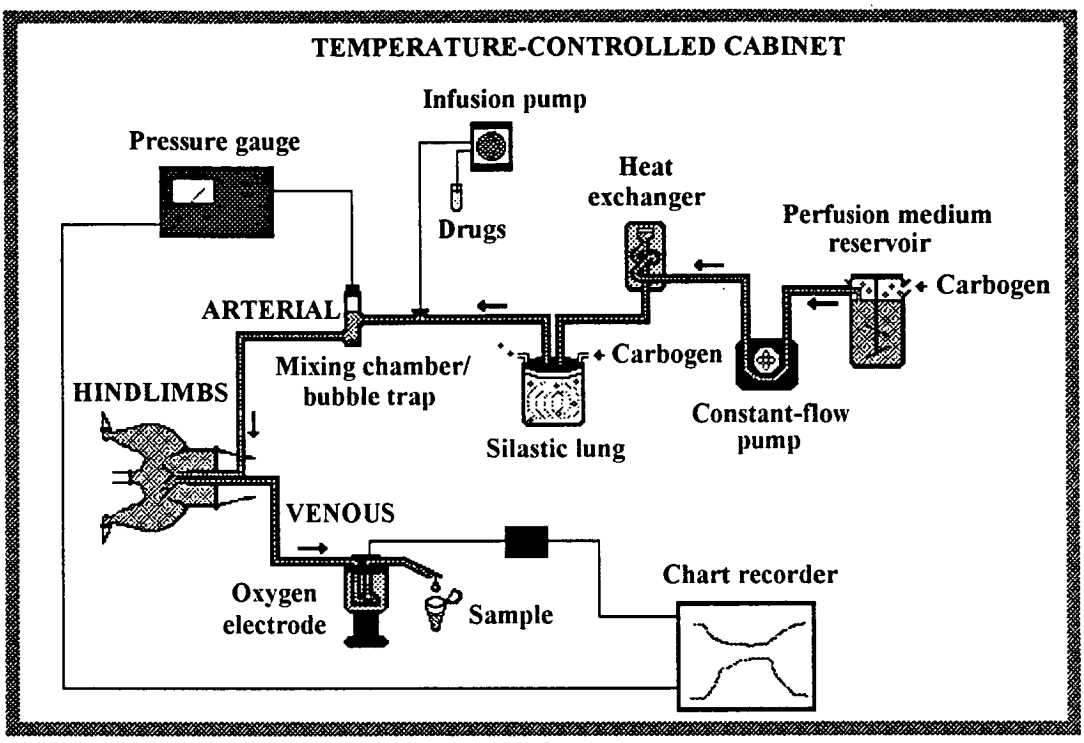

Figure 2.3 Rat hindlimb perfusion apparatus.

Carbogen-gassed perfusion medium was pumped at a constant-flow rate by a peristaltic pump from the reservoir through the apparatus. Before reaching the hindlimb, the medium passed through a water-jacketed glass heat exchange coil, and a carbogen-gassed silastic tubing oxygenator. Perfusion pressure was continuously monitored in a sidearm mixing chamber/bubble trap proximal to the aorta. Continuous measurement of venous effluent oxygen content was achieved by an in-line 0.5-ml Clark-type oxygen electrode contained in a temperature-controlled water-jacket. Venous effluent was periodically sampled for measurement of lactate and glucose concentrations. Carbogen, 95% O_2-5% CO_2.

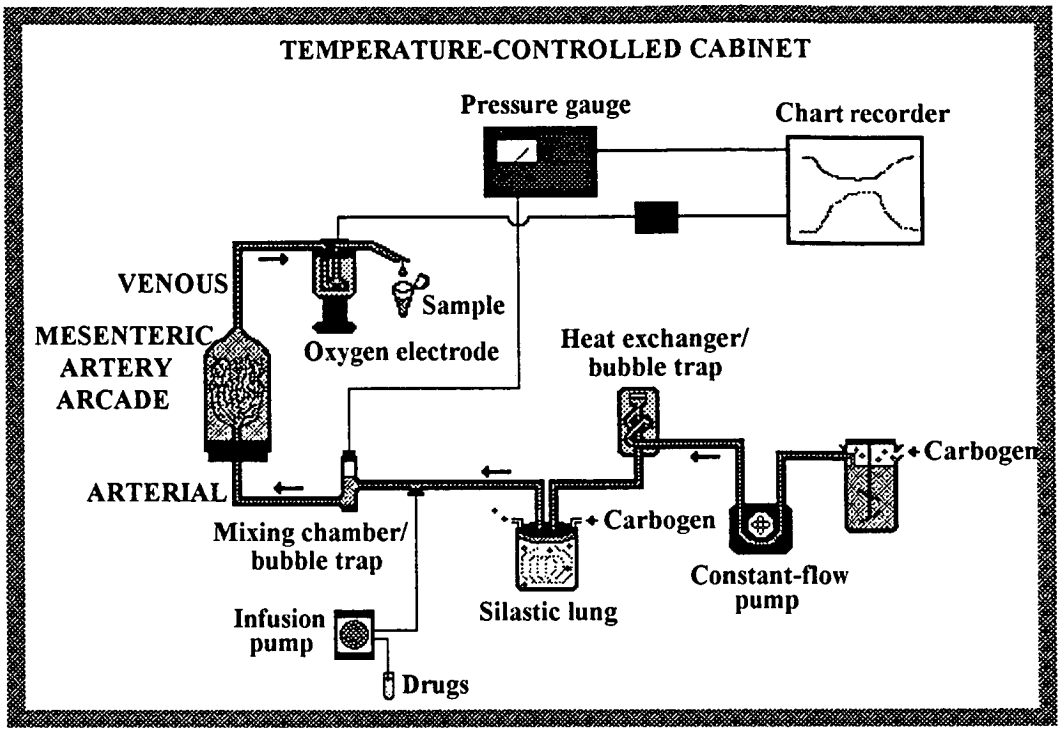

Figure 2.4 Rat mesenteric artery arcade perfusion apparatus.

Carbogen-gassed perfusion medium was pumped at a constant-flow rate by a peristaltic pump from the reservoir through the apparatus. Before reaching the mesenteric artery arcade, the medium passed through a water-jacketed glass heat exchange coil, and a carbogen-gassed silastic tubing oxygenator. Perfusion pressure was continuously monitored in a sidearm mixing chamber/bubble trap proximal to the artery. Continuous measurement of venous effluent oxygen content was achieved by an in-line 0.5-ml Clark-type oxygen electrode contained in a temperature-controlled water-jacket. Venous effluent was periodically sampled for measurement of lactate concentrations. Carbogen, 95% O_2-5% CO_2.

silastic tubing (ID 1.47 mm/OD 1.91 mm, Dow Corning, USA) which was continually gassed with 95% O_2-5% CO_2. This ensured constant arterial PO_2 levels.

Perfusion pressure was continuously monitored in a sidearm proximal to the aorta. Changes in perfusion pressure reflected changes in vascular resistance.

Continuous measurement of venous effluent oxygen content was achieved by an in-line 0.5-ml Clark-type oxygen electrode contained in a temperature-controlled water-jacket (usually 25°C).

Arterial PO_2 at the perfusion flow rate was measured before and after completion of experiments. Calibration of the oxygen electrode was made routinely by using temperature-equilibrated 100% O_2, air, and N_2 before and after each experiment. The electrode response was assumed to be linear in the range of PO_2 200-700 mmHg, with only an error of <10% in calculating oxygen uptake when compared to the slight curve obtained using various mixtures of O_2-N_2.

Freshly prepared solutions of agonists and antagonists were infused continuously (LKB Brommer, 2232 Microprepex S) into the perfusion line proximal to a small, stirred bubble trap and the arterial cannula. An infusion rate of <2% of the flow rate ensured the vehicle had no apparent effect on venous PO_2 or perfusion pressure.

2.2.1.5 Chemicals and drugs.

(-)-Norepinephrine bitartrate, 5-hydroxytryptamine creatinine sulfate, prazosin HCl, (±)-propranolol HCl, (-)-isoproterenol HCl, arginine vasopressin, human angiotensin II, ethyleneglycol-bis-(b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and hydrazinium hydrate from Sigma, USA; ketanserin tartrate, LY 53,857 maleate, 5-methyl urapidil, chloroethylclonidine 2HCl, and WB-4101 HCl from Research Biochemicals Inc., USA; porcine insulin (Actrapid MC, 100 units/ml) was obtained from Novo Industri A/S; 2-deoxy-D-[1-^{3}H]glucose and [U-^{14}C]sucrose were obtained from Amersham Australia Pty Ltd.; BSA (fraction V), pentobarbital sodium, lactate dehydrogenase and nicotinamide adenine dinucleotide from Boehringer Mannheim, Australia. Heparin was from David Bull, Australia. Filtration apparatus (model RG5) from Amicon and millipore filters (0.45 μm) from Millipore Corp., USA. All other chemicals were of analytical grade from Ajax, Australia. 95% O_2-5% CO_2 (carbogen) and 95% N_2-5% CO_2 gas mixtures from CIG, Australia.

2.2.1.6 Oxygen uptake calculations.

Oxygen uptake rates ($\dot{V}O_2$) for hindlimbs perfused with erythrocyte-free medium were calculated by multiplying the arteriovenous difference (A-V) in oxygen content by the flow rate of the perfusate per gram of hindlimb skeletal muscle (SKM). $\dot{V}O_2$ in $\mu moles.h^{-1}.g^{-1}$ was calculated as follows:

$$\underset{(\mu moles.h^{-1}.g^{-1})}{\dot{V}O_2} = \frac{(A\text{-}V) \times 0.03388^{*} \times 60 \times \text{flow rate } (ml.min^{-1})}{\text{SKM mass}^{\dagger} \times 22.4^{\#}}$$

* µl of oxygen dissolved per 100 µl of perfusate medium per mmHg PO_2 at 25°C (Bunsen coefficient) from Christoforides *et al.* (1969).

† Assumed to be one twelfth the rat body mass per hindlimb (Ruderman *et al.*, 1971).

\# At standard temperature and pressure the volume of 1 µmol is 22.4 µl.

Calculation of $\dot{V}O_2$ by the perfused mesenteric artery arcade preparation was performed in a manner similar to that for the hindlimb. The perfused mass was determined upon completion of each experiments (approx. 1 g) and used in the calculation. It was estimated that vascular tissue comprised approx. 6% of the perfused tissue (Ye *et al.*, 1990a).

2.2.1.7 Dose-response curves.

When generating dose-response curves for agonists, venous PO_2 and perfusion pressure values were taken after 15 min of agonist infusion when steady-state values were achieved. In some experiments, perfusate samples were collected for lactate analysis. In general, perfusate samples were pooled for the 15 min of drug infusion to give a mean value of lactate concentration, except for time courses when 1 ml samples were collected at the times indicated.

2.2.1.8 Statistical analysis.

Data are shown as the means % standard errors. Curves were fitted using the Sigma Plot program (Version 4.0, Jandel Scientific).

The statistical significance of differences between groups of data was assessed by the either the paired or unpaired 2-tailed Student's *t* test or one way analysis of variance and least significant difference analysis (Snedecor & Cochran, 1980).

2.2.2 Metabolite assay techniques.

In some experiments venous effluent was assayed for lactate concentration. Perfusate samples were usually taken when steady state was reached as judged by pressure and $\dot{V}O_2$. Samples were collected into eppendorf tubes and kept at -20°C. Immediately prior to performing the assay, 0.1 ml of 2 M perchloric acid was added to 0.5 ml of perfusate sample, mixed and centrifuged in an Eppendorf microfuge for 5 min at 2°C. An aliquot (30 µl) of 2.5 M K_2CO_3 was added to 0.4 ml of supernatant, mixed and centrifuged for 5 min as above. The neutralized supernatant was then used for the lactate assays.

The method used was that of Gutmann and Wahlefeld (1974). Absorbance before and after lactate dehydrogenase addition was measured spectrophotometrically at 340 nm. The net change in absorbance (ΔE) was used to calculate the concentration of lactate in the sample as follows:

$$[\text{lactate}]_{(\text{mM})} = \Delta E \times \frac{1000}{6220^*} \times \frac{\text{ml (cell vol.)}}{\text{ml (neutralized vol.)}} \times \frac{\text{ml (total vol.)}}{\text{ml (perfusate vol.)}}$$

* Extinction coefficient

2.3 Results.

In this series of experiments the steady state mean perfusate arterial and venous P_{O_2} values for the rat hindlimb were 683.4 ± 4.4 mmHg and 399.9 ± 6.7 mmHg, respectively (*n* = 68). These values were obtained after an initial 30 min equilibration period. The basal $\dot{V}O_2$ by hindlimb muscle was calculated as previously described (Section 2.2.1.6) and, using the above values, was found to be 6.5 ± 0.1 µmol.h^{-1}.g^{-1} (*n* = 68). Steady state basal lactate production was 7.4 ± 0.5 µmol.h^{-1}.g^{-1} (*n* = 23) and basal perfusion pressure was 22.3 ± 0.4 mmHg (*n* = 68). The steady state basal values for $\dot{V}O_2$, perfusion pressure and lactate release remained constant for the duration of control experiments (vehicle only) and were similar to those

reported previously from this laboratory (Colquhoun *et al.*, 1988, 1990; Clark *et al.*, 1990; Richards *et al.*, 1992, 1993; Hettiarachchi *et al.*, 1992).

2.3.1 Effects of NE in the perfused hindlimb.

Upon NE infusion in the range 1-250 nM, $\dot{V}O_2$, lactate release and perfusion pressure increased dose-dependently (Figure 2.7). A dose of 50 nM NE was chosen to represent a stimulatory effect on $\dot{V}O_2$ (Figures 2.5 & 2.6). This dose decreased venous PO_2 by 167.9 ± 20.7 mmHg, equivalent to an (almost maximal) increase in $\dot{V}O_2$ of 4.1 ± 0.5 $\mu mol.h^{-1}.g^{-1}$, associated with an increase in perfusion pressure above basal of 28.6 ± 0.7 mmHg (n = 5). In addition, NE rapidly stimulated a transient, 4-fold increase in hindlimb lactate release, which reached steady state 20 min after infusion to a value 12.9 ± 1.6 $\mu mol.h^{-1}.g^{-1}$ above basal (Figure 2.6, n = 3). It was noted that upon infusion of NE, the presence of a small quantity of erythrocytes transiently appeared in the perfusate.

NE, at 50 nM, appeared to stimulate predominantly α_1-ARs, as prazosin was approximately 100-fold more potent in blocking the NE-induced response than the α_2-AR-antagonist, yohimbine (Figure 2.8). 0.1 μM prazosin almost totally opposed the 50 nM NE-mediated effects. This dose of NE had low affinity for the β-AR (Figure 2.10).

At doses tested beyond 0.25 μM NE, perfusion pressure continued to increase, but steady state $\dot{V}O_2$ and lactate release decreased dose dependently towards basal levels (Figure 2.7). After an initial, transient stimulation of $\dot{V}O_2$ (Figure 2.5), 2.5 μM NE significantly inhibited $\dot{V}O_2$ to levels 0.5 ± 0.2 $\mu mol.h^{-1}.g^{-1}$ below basal, with a corresponding pressure increase of 206.0 ± 6.2 mmHg (n = 16, Figure 2.7). This higher dose of NE appeared to stimulate both α_1- and β-ARs. Lower doses of prazosin selectively blocked the vasoconstriction associated with the inhibitory component of $\dot{V}O_2$ (Figures 2.5 & 2.9). A dose of 0.5 μM prazosin was required to oppose the 2.5 μM NE-mediated effects on perfusion pressure and $\dot{V}O_2$ (Figure 2.9). Similar to the low-dose NE effects, a 100-fold higher dose of yohimbine was required to block the vasoconstriction-associated effects, although the antagonist dose-range was narrower (Figure 2.9). Propranolol increased high-dose NE-mediated perfusion pressure and $\dot{V}O_2$ (Figure 2.10), although not significantly. Interestingly, ketanserin also selectively blocked the inhibition of $\dot{V}O_2$ and the part of perfusion pressure increase (Figure 2.9), presumably due to antagonism of α_1-AR, not 5-HT_{2A}-

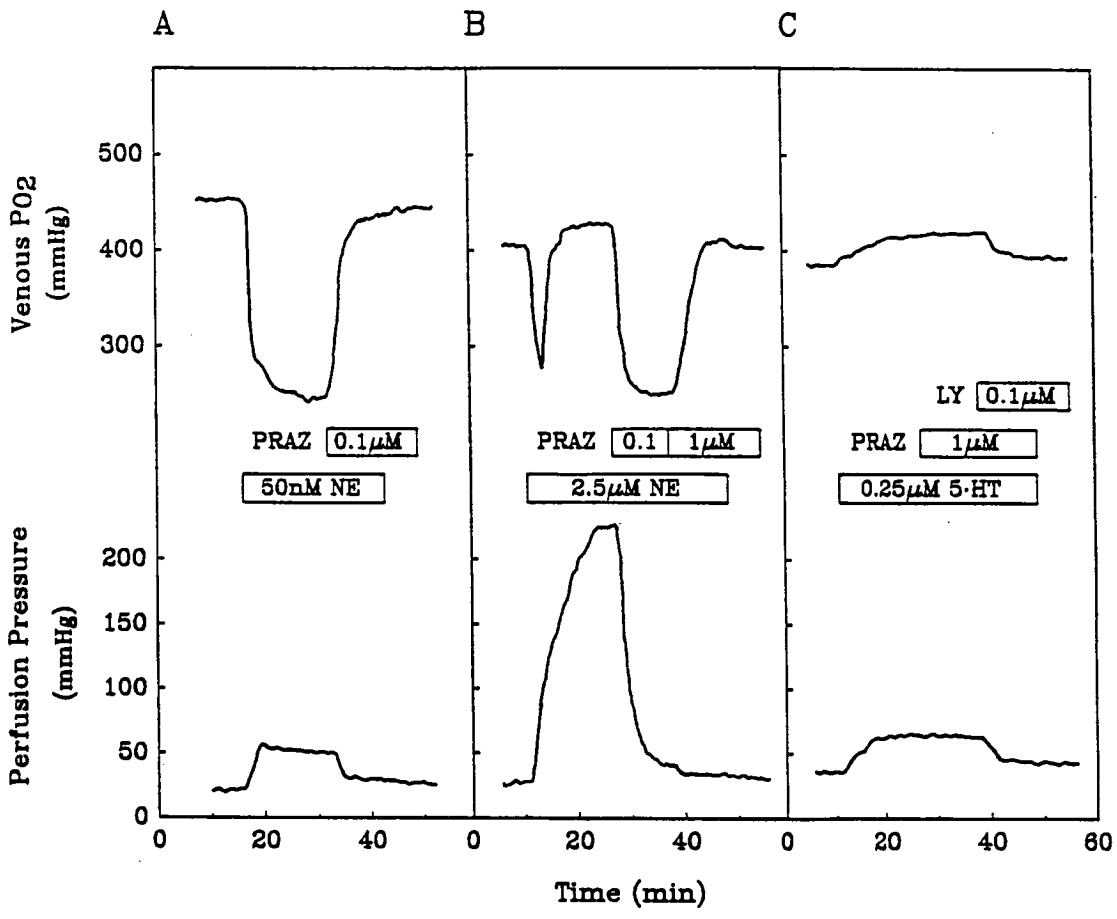

Figure 2.5 Effects of representative low- and high-dose NE and 5-HT concentrations on hindlimb venous PO_2 and perfusion pressure.

Hindlimbs from 180-200 g rats were perfused at 25°C and a constant flow rate of 4 $ml.min^{-1}$ with perfusion medium containing 2% bovine serum albumin and equilibrated against 95% O_2-5% CO_2. Additions were 50 nM NE and 0.1 μM prazosin (PRAZ, *A*), 2.5 μM NE, 0.1 μM and 1 μM prazosin (*B*), and 0.25 μM 5-HT, 1 μM prazosin and 0.1 μM LY 53,857 (LY, *C*). NE, norepinephrine; 5-HT, serotonin.

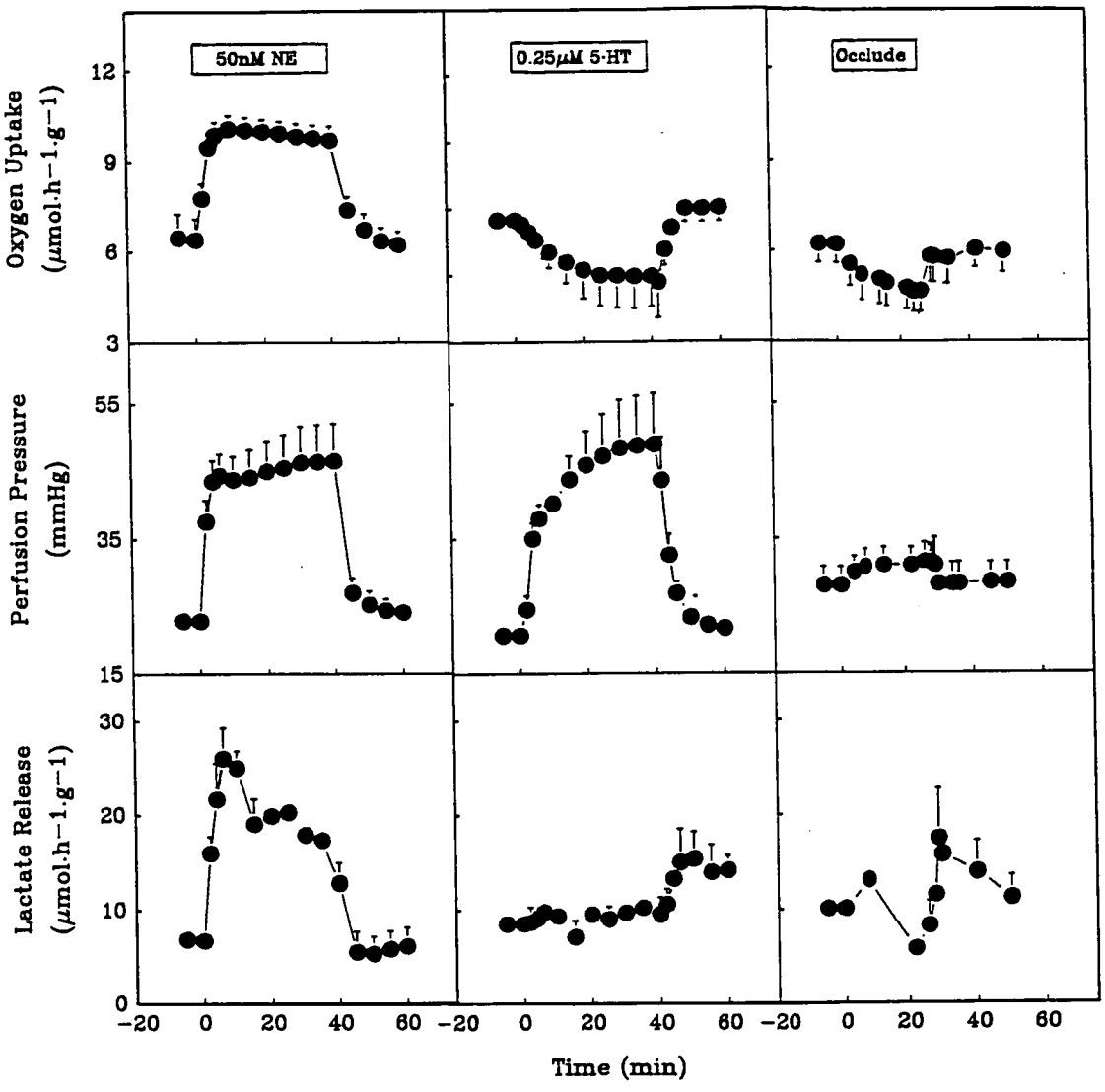

Figure 2.6 **Time course for the effects of NE, 5-HT, and reversible occlusion at the knee on hindlimb $\dot{V}O_2$, perfusion pressure and lactate release.**

Hindlimbs were perfused as described in Figure 2.5. Treatments were 50 nM NE, 0.25 μM 5-HT and reversible hindlimb occlusion at the knee (Occlude). Values are means ± SE for 3 hindlimbs, when not visible, error bars are within symbols. NE, norepinephrine; 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

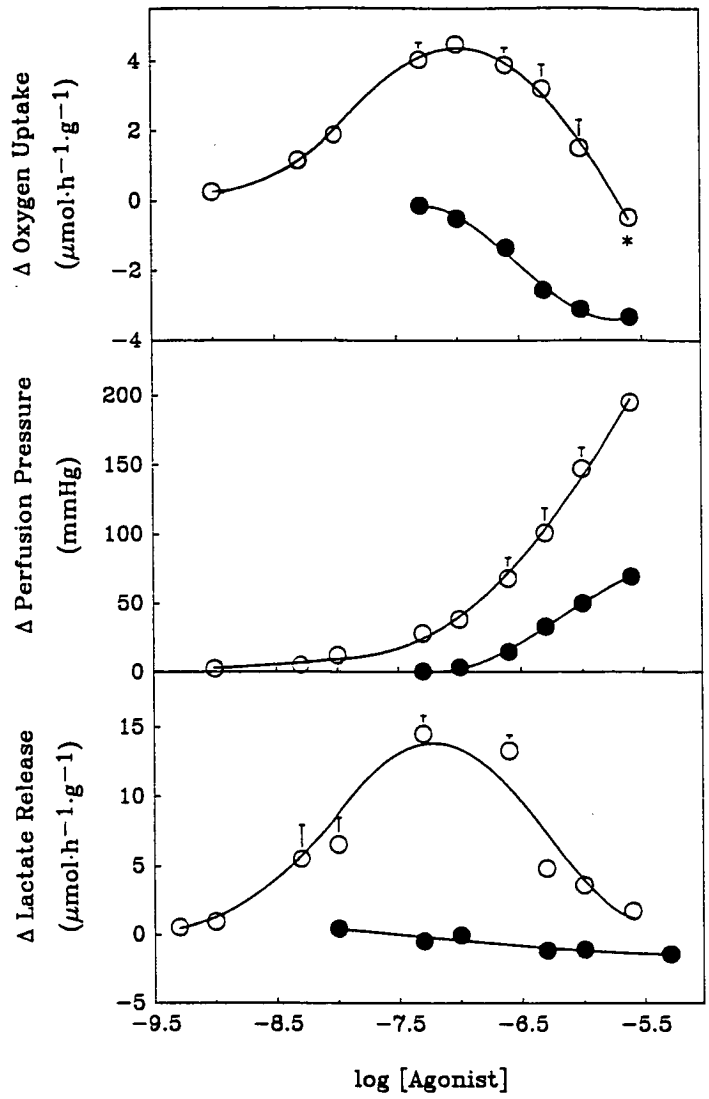

Figure 2.7 Dose-response curves for NE and 5-HT on changes in hindlimb oxygen uptake, perfusion pressure and lactate release.

Hindlimbs were perfused as described in Figure 2.5. Basal arterial and venous PO_2 were 694.9 ± 8.5 and 416.6 ± 12.9 mmHg respectively, equating to a basal $\dot{V}O_2$ of 6.4 ± 0.2 μmol.h^{-1}.g^{-1} (n = 24). Basal perfusion pressure was 24.1 ± 0.7 mmHg (n = 24) and basal lactate release was 6.7 ± 0.7 μmol.h^{-1}.g^{-1} (n = 13). Steady state values for each dose were measured after 15 min of either NE (O) or 5-HT (●) infusion. Values are means ± SE for at least 4 hindlimbs. When not visible, error bars are within symbols. * $P < 0.05$, 2.5 μM NE-induced $\dot{V}O_2$ below basal, paired Student's t test (n = 16). NE, norepinephrine; 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

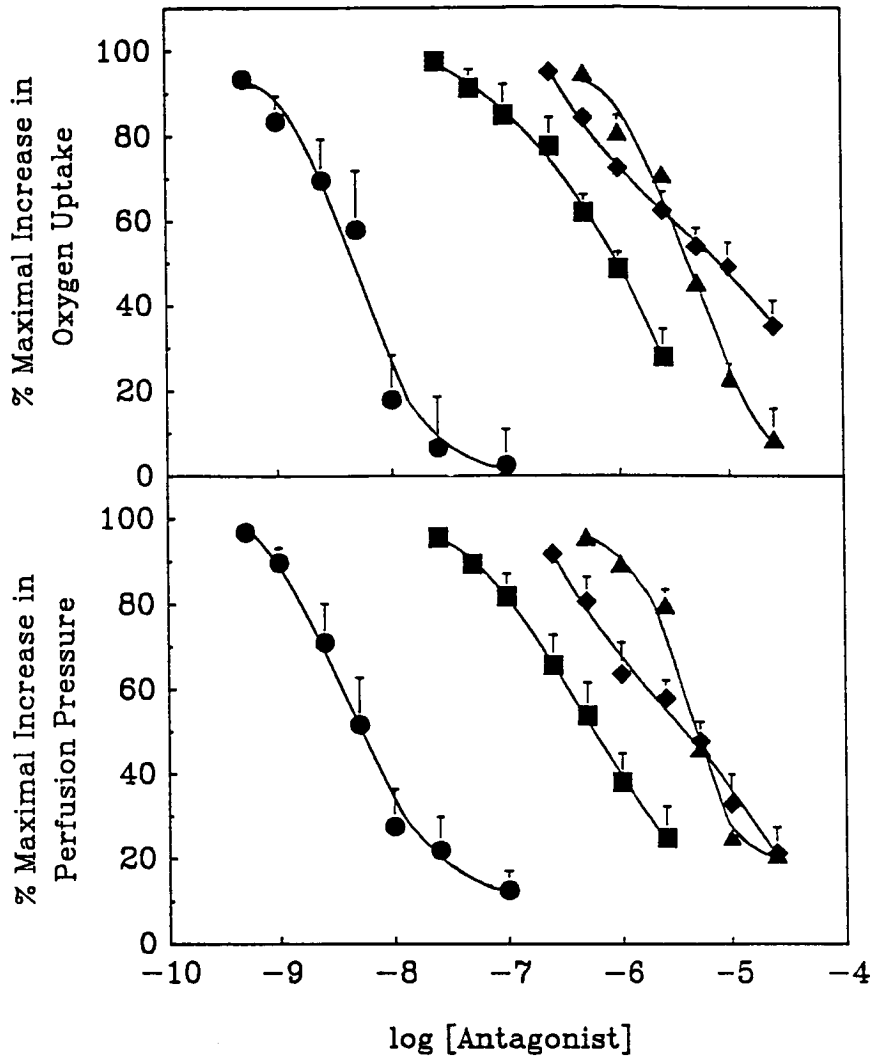

Figure 2.8 Effect of α-AR antagonists on the response to low-dose NE on hindlimb $\dot{V}O_2$ and perfusion pressure.

Hindlimbs were perfused as described in Figure 2.5. Basal arterial and venous PO_2 were 675.6 ± 7.8 and 402.4 ± 12.4 mmHg respectively, equating to a basal $\dot{V}O_2$ of 6.4 ± 0.3 $\mu mol.h^{-1}.g^{-1}$ (n = 12). Basal perfusion pressure was 21.9 ± 1.1 mmHg (n = 12). After 15 min of infusion, 50 nM NE increased $\dot{V}O_2$ by 3.8 ± 0.2 $\mu mol.h^{-1}.g^{-1}$, and perfusion pressure by 22.4 ± 1.2 mmHg (n = 12). Increasing concentrations of each antagonist were then infused: prazosin (●); yohimbine (▲); ketanserin (■) and LY 53,857 (◆). Values are means ± SE for 3 hindlimbs with each treatment, expressed as a percentage of the maximal NE response for each hindlimb. When not visible, error bars are within symbols. AR, adrenoceptor; NE, norepinephrine; $\dot{V}O_2$, oxygen uptake.

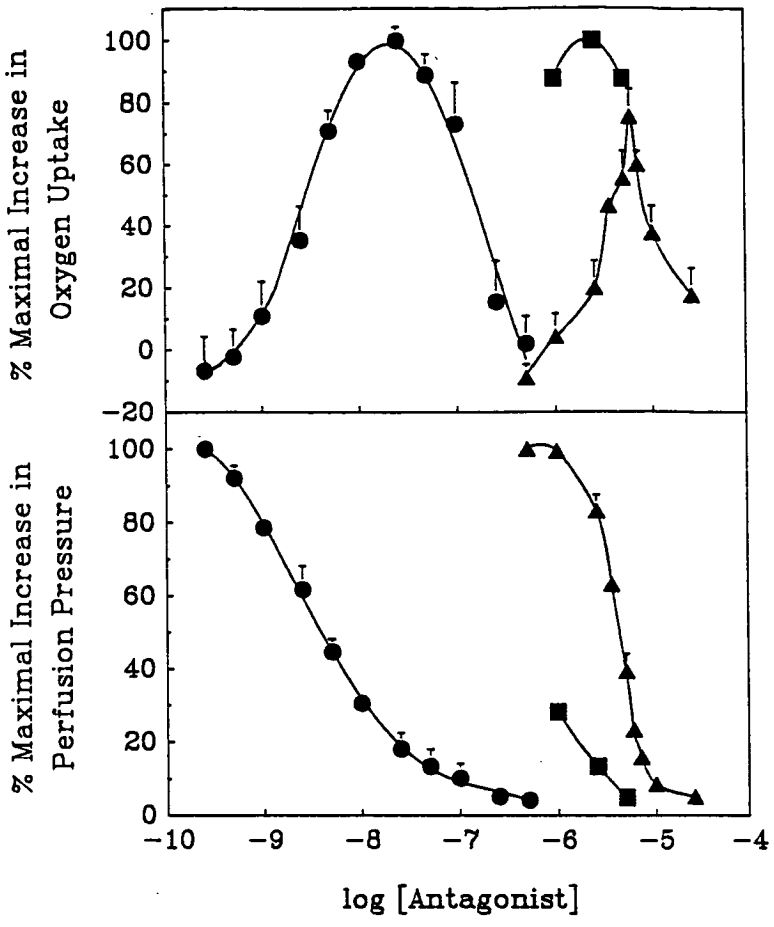

Figure 2.9 Effect of α-AR antagonists on the response to high-dose NE on hindlimb $\dot{V}O_2$ and perfusion pressure.

Hindlimbs were perfused as described in Figure 2.5. Basal arterial and venous PO_2 were 700.8 ± 8.7 and 405.3 ± 9.2 mmHg respectively, equating to a basal $\dot{V}O_2$ of 6.7 ± 0.2 μmol.h^{-1}.g^{-1} (n = 9). Basal perfusion pressure was 21.8 ± 0.9 mmHg (n = 9). After 15 min of infusion, 2.5 μM NE decreased $\dot{V}O_2$ by 0.4 ± 0.3 μmol.h^{-1}.g^{-1}, and increased perfusion pressure by 189.9 ± 7.0 mmHg (n = 9). Increasing concentrations of each antagonist were then infused: prazosin (●); yohimbine (▲) and ketanserin (■). Data are expressed as a percentage of the maximal increase in $\dot{V}O_2$ (4.1 μmol.h^{-1}.g^{-1}) and perfusion pressure (206 mmHg) mediated by NE . Values are means ± SE for 3 hindlimbs with each treatment. When not visible, error bars are within symbols. AR, adrenoceptor; NE, norepinephrine; $\dot{V}O_2$, oxygen uptake.

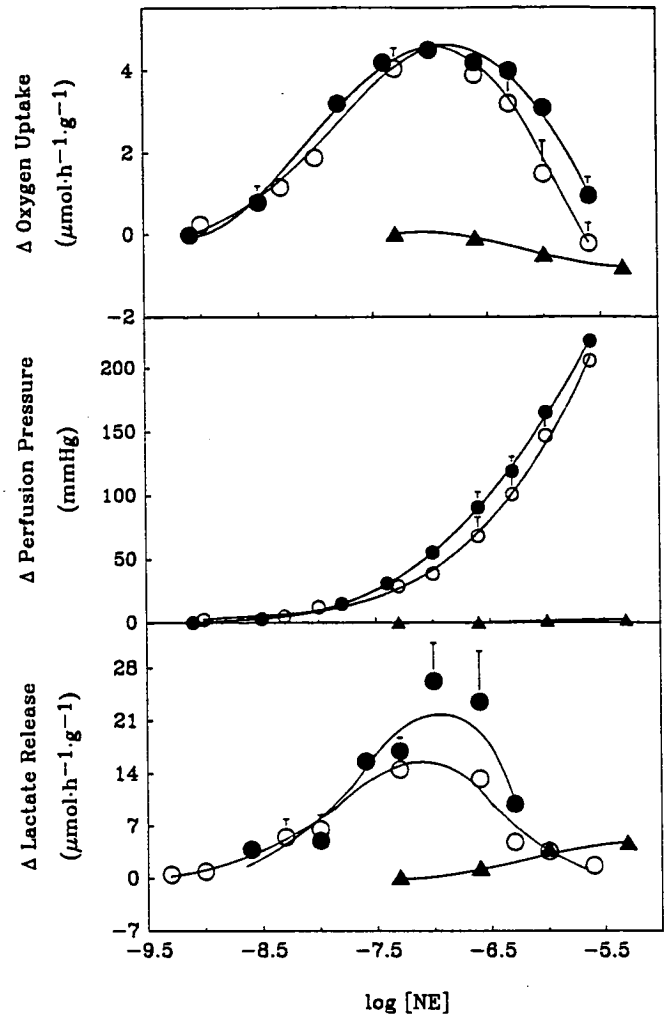

Figure 2.10 Effect of α_1- and β-AR antagonists on the NE dose-response curve on changes in hindlimb $\dot{V}O_2$, perfusion pressure and lactate release.

Hindlimbs were perfused as described in Figure 2.5. Basal arterial and venous PO_2 were 696.6 ± 7.0 and 429.8 ± 8.6 mmHg respectively, equating to a basal $\dot{V}O_2$ of 6.2 ± 0.2 μmol.h^{-1}.g^{-1} (n = 28). Basal perfusion pressure was 24.6 ± 0.5 mmHg (n = 28), and lactate release 6.9 ± 0.8 μmol.h^{-1}.g^{-1} (n = 14). NE dose-response curves were obtained in the absence (O) or presence of 10 μM (±)-propranolol (●) or 2.5 μM prazosin (▲). Antagonists were infused for at least 15 min prior to commencement NE infusion. Values are means ± SE for at least 3 hindlimbs, when not visible, error bars are within symbols. AR, adrenoceptor; NE, norepinephrine; $\dot{V}O_2$, oxygen uptake.

receptors, as ketanserin, but not prazosin, blocked the 5-HT-mediated response (Section 2.3.2).

Once the α_1-ARs were blocked with prazosin, NE dose-dependently decreased $\dot{V}O_2$ and stimulated lactate release associated with almost no change in perfusion pressure (Figure 2.10). These responses were qualitatively similar to that of the β-agonist isoproterenol, although NE appeared to exhibit a lower β-AR affinity (approx. 40-fold less, Figure 2.11).

2.3.2 Effects of 5-HT in the perfused hindlimb.

5-HT dose-dependently increased perfusion pressure and decreased $\dot{V}O_2$ (Figure 2.7). A dose of 0.25 μM 5-HT increased perfusion pressure by 26.1 ± 3.2 mmHg, a level comparable to that stimulated by 50 nM NE. However in contrast to NE, 0.25 μM 5-HT increased venous PO_2 by 62.2 ± 8.3 mmHg, equivalent to a decrease in $\dot{V}O_2$ of 1.5 ± 0.2 $\mu mol.h^{-1}.g^{-1}$ (n = 8), and decreased basal hindlimb lactate release by 0.2 ± 0.8 $\mu mol.h^{-1}.g^{-1}$ (n = 6, Figures 2.5 & 2.6). Upon removal of 5-HT a transient increase in lactate release of 7.6 ± 2.6 $\mu mol.h^{-1}.g^{-1}$ above basal was observed, which gradually returned towards basal. Another distinguishing feature of the 5-HT response was the absence of erythrocyte washout from the hindlimb during vasoconstriction.

5-HT appeared to stimulate predominantly $5\text{-}HT_{2A}$-receptors, as ketanserin (0.1 μM) (data not shown) and LY 53,857 (2.5 nM) almost totally blocked the 5-HT-mediated responses on perfusion pressure, $\dot{V}O_2$ (Figure 2.12) and lactate release (data not shown). LY 53,857 was chosen to inhibit the 5-HT response as it had lower affinity for the α-AR (Figure 2.8). Prazosin (1 μM) had no significant effect on the 5-HT response (Figure 2.5). Isoproterenol (50 nM) inhibited 19 ± 11 % and 20 ± 7 % of the $\dot{V}O_2$ and perfusion pressure responses, respectively, to 0.25 μM 5-HT (n = 3).

Once the $5\text{-}HT_{2A}$-receptor stimulation was fully blocked with 10 nM LY 53,857, 0.25 μM 5-HT caused a slight vasodilatation of approx. 1 mmHg, and a decrease in $\dot{V}O_2$ of 0.5 ± 0.1 $\mu mol.h^{-1}.g^{-1}$ (n = 2). Also in the presence of 10 nM LY 53,857, 5-HT dose-dependently opposed the response to 5 nM angiotensin II (Figures 2.13 & 2.14).

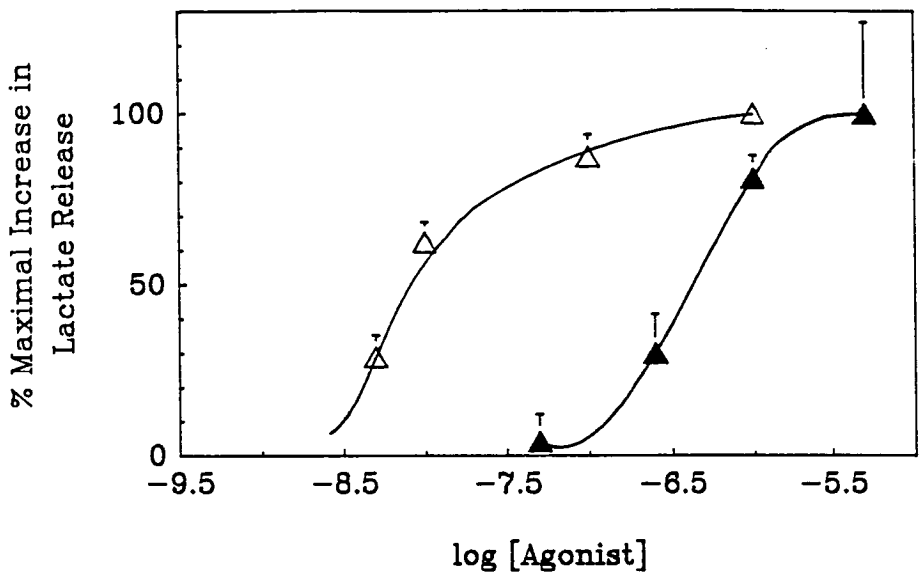

Figure 2.11 Dose-response curves for β-agonists on hindlimb lactate release expressed as a proportion of the maximum response by each agonist.

Hindlimbs were perfused as described in Figure 2.5. NE plus 2.5 μM prazosin (▲) data was taken from Figure 2.10; isoproterenol (△) data from Hettiarachchi *et al.* (1992). Maximal doses of NE (5 μM) and isoproterenol (1 μM) increased lactate release by 4.8 ± 1.3 $\mu mol.h^{-1}.g^{-1}$ (n = 3) and 6.4 ± 0.1 $\mu mol.h^{-1}.g^{-1}$ (n = 5), respectively. Values are means ± SE, expressed as a percentage of the maximal agonist response for each hindlimb. When not visible, error bars are within symbols.

2.2.3 *Effects of reversible hindlimb occlusion at the knee.*

Physical, reversible occlusion of flow to the lower hindlimb was achieved by tying a soft, rubber tourniquet below the knee. By dissection of tissue stained with injected Evans Blue dye, it was found that occlusion directly removed approx. 2.5 g (16.7%) of muscle from the circulation and restricted perfusion to a further irregular band of tissue immediately above the ligature that could not be accurately quantified but could represent up to an additional 10%. After 15 min of hindlimb occlusion, perfusion pressure was increased by 3.5 ± 0.6 mmHg, $\dot{V}O_2$ decreased by 1.5 ± 0.3 $\mu mol.h^{-1}.g^{-1}$, (n = 4) and lactate release decreased by 1.8 ± 1.1 $\mu mol.h^{-1}.g^{-1}$ (n = 2, Figure 2.6). Upon removal of the tourniquet, oxygen and pressure levels rapidly returned to basal, and a transient peak of lactate production was observed.

2.3.4 *Effects of 5-HT in the perfused mesenteric artery arcade.*

In the isolated, perfused rat mesenteric artery arcade, steady state mean PO_2 values were 628 ± 34 mmHg arterial and 574 ± 36 mmHg venous. The basal $\dot{V}O_2$ was 7.2 ± 0.7 $\mu mol.h^{-1}.g^{-1}$, and basal perfusion pressure 16 ± 5 mmHg (n = 10). These values were similar to those previously reported (Ye *et al.*, 1990a). The basal lactate release was 5.5 ± 0.6 $\mu mol.h^{-1}.g^{-1}$ (n = 10). The contribution to basal metabolism of non-vascular tissue supplied by the vessels of the mesenteric artery preparation was assessed by infusing both the vasodilators nitroprusside (0.5 mM) and isoproterenol (1 μM). Under these conditions $\dot{V}O_2$ was found to be negligible (data not shown) suggesting minimal metabolism by the surrounding lymph and fat tissue.

The effect of NE in the mesenteric artery has been reported previously and were qualitatively similar to those observed with 5-HT (Ye *et al.*, 1990b). 5-HT dose-dependently increased $\dot{V}O_2$, perfusion pressure and lactate release (Figure 2.16). The maximum dose of 5-HT (6.7 μM) increased $\dot{V}O_2$ by 9.1 ± 0.8 $\mu mol.h^{-1}.g^{-1}$, perfusion pressure by 11 ± 2 mmHg and lactate release by 6.6 ± 0.8 $\mu mol.h^{-1}.g^{-1}$ (n = 5, Figure 2.16). Nitroprusside (0.5 mM) blocked the 5-HT-mediated effects on perfusion pressure and $\dot{V}O_2$ (Figure 2.15). Ketanserin (0.7 μM) totally inhibited the response to 6.7 μM 5-HT, and had no effect on that of NE (data not shown).

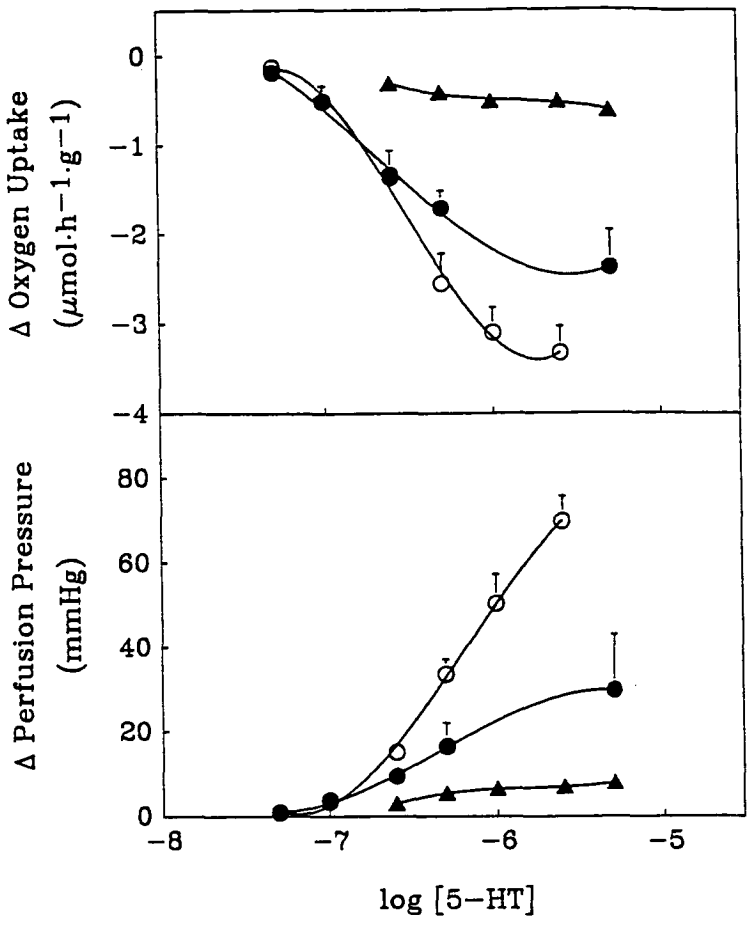

Figure 2.12 Effect of a 5-HT_2-antagonist on the 5-HT dose-response curve on changes in hindlimb $\dot{V}O_2$ and perfusion pressure.

Hindlimbs from 180-200 g rats were perfused at 25°C and a constant flow rate of 4 $ml.min^{-1}$ with perfusion medium containing 2% bovine serum albumin and equilibrated against 95% O_2-5% CO_2. Basal arterial and venous PO_2 were 639.2 ± 8.9 and 306.4 ± 4.5 mmHg respectively, equating to a basal oxygen uptake of 7.5 ± 0.3 $\mu mol.h^{-1}.g^{-1}$ (n = 8). Basal perfusion pressure was 17.8 ± 0.3 mmHg (n = 8). 5-HT was infused in the absence (O) or presence of either 0.5 nM (●) or 2.5 nM LY 53,857 (▲). LY 53,857 was infused for 30 min prior to commencement of 5-HT infusion. Values are means ± SE from at least three hindlimbs; where not visible, error bars are within symbols. 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

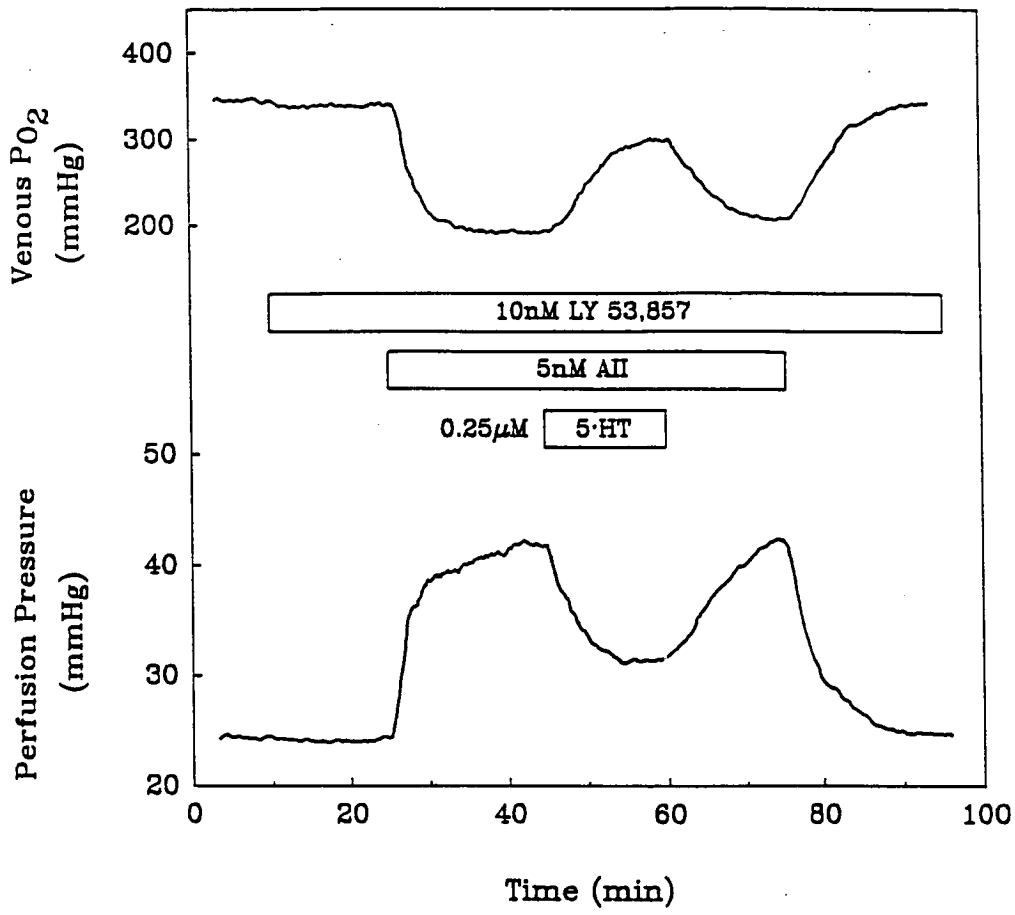

Figure 2.13 Effect of a representative dose of 5-HT in the presence of both a 5-HT_2-antagonist and angiotensin II on hindlimb venous PO_2 and perfusion pressure.

Hindlimbs were perfused as described in Figure 2.12. 5-HT (0.25 μM) was infused in the presence of LY 53,857 (10 nM) and angiotensin II (AII, 5 nM). Values are means ± SE from three hindlimbs; where not visible, error bars are within symbols. 5-HT, serotonin.

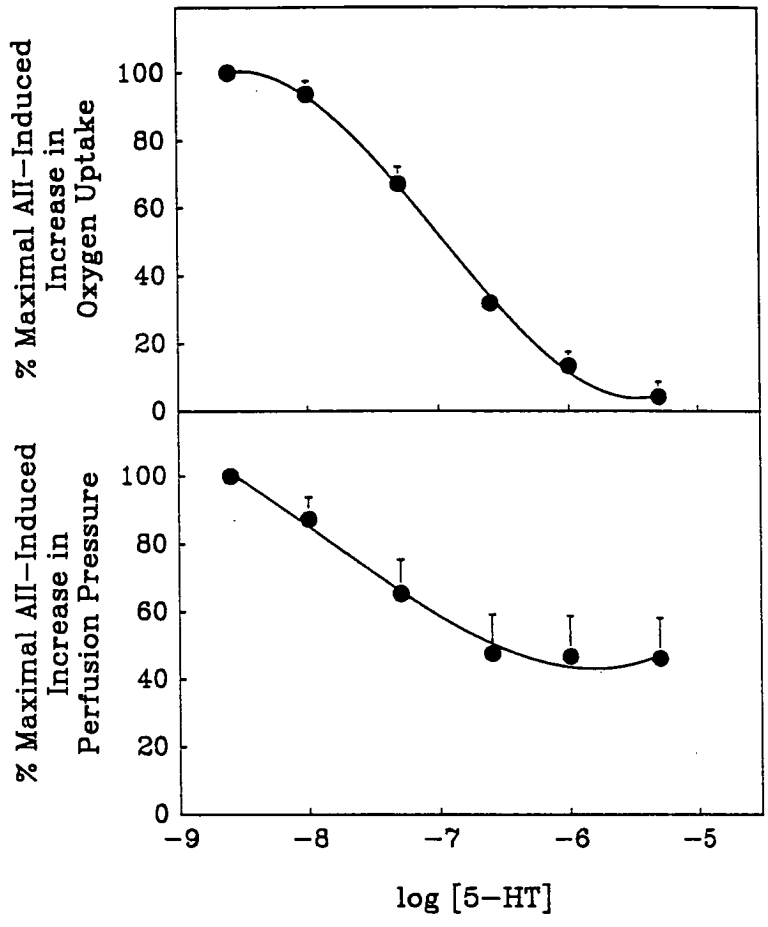

Figure 2.14 Dose response curve for 5-HT in the presence of both a 5-HT_2-antagonist and angiotensin II on hindlimb $\dot{V}O_2$ and perfusion pressure.

Hindlimbs were perfused as described in Figure 2.12. Basal arterial and venous PO_2 were 690.9 ± 6.5 and 344.4 ± 29.4 mmHg respectively, equating to a basal $\dot{V}O_2$ of 8.0 ± 0.4 $\mu mol.h^{-1}.g^{-1}$ (n = 3). Basal perfusion pressure was 20.0 ± 0.6 mmHg (n = 3). 5-HT was infused in the presence of LY 53,857 (10 nM) and angiotensin II (5 nM). Values are expressed as percentages of the maximal mean ± SE AII responses; where not visible, error bars are within symbol. 5 nM angiotensin II increased $\dot{V}O_2$ by 3.9 ± 0.4 $\mu mol.h^{-1}.g^{-1}$ and perfusion pressure by 23.8 ± 8.8 mmHg (n = 3). 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

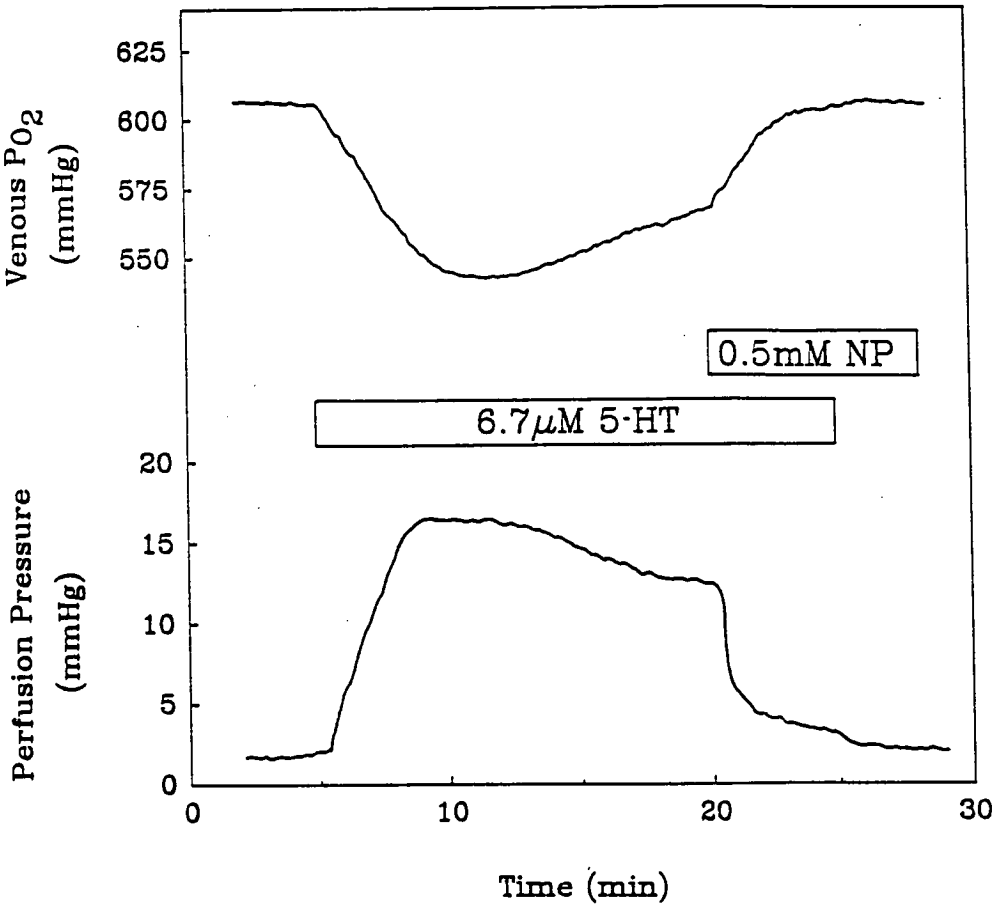

Figure 2.15 Time course of 5-HT-mediated effects on venous PO_2 and perfusion pressure in isolated perfused mesenteric artery arcade.

Mesenteric artery arcades from 200-250 g rats were perfused at 25°C and a constant flow rate of 1.5 $ml.min^{-1}$ with perfusion medium containing 2% bovine serum albumin and equilibrated against 95% O_2-5% CO_2. 5-HT, serotonin; NP, nitroprusside.

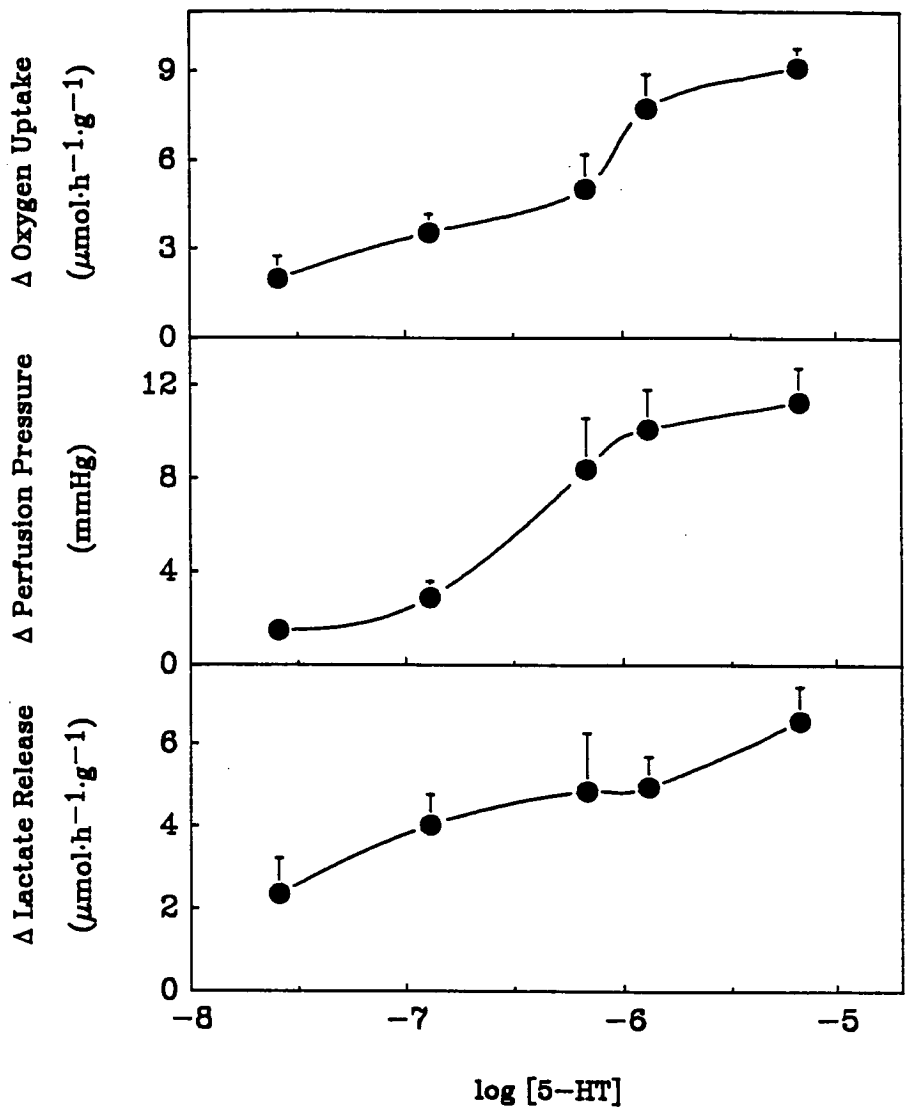

Figure 2.16 Dose-response curves for 5-HT on changes in $\dot{V}O_2$, perfusion pressure and lactate release in isolated perfused mesenteric artery arcade.

Mesenteric artery arcades were perfused as described in Figure 2.15. Basal arterial and venous P_{O_2} were 628 ± 34 mmHg and 628 ± 34 mmHg, equivalent to an $\dot{V}O_2$ of 7.1 ± 0.7 μmol.h^{-1}.g^{-1}, and basal perfusion pressure was 25 ± 8 mmHg (n = 10). Values are means ± SE for a minimum of 4 perfusions. When not visible, error bars are within symbols. 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

2.4 Discussion.

It has previously been shown that in the constant-flow perfused rat hindlimb NE, angiotensin II and vasopressin stimulated dose-dependent increases in perfusion pressure and associated increases in $\dot{V}O_2$ and lactate release (Colquhoun *et al.*, 1988; Richards *et al.*, 1991; Hettiarachchi *et al.*, 1992). These effects were blocked by the smooth muscle vasodilator nitroprusside (Colquhoun *et al.*, 1988; Hettiarachchi *et al.*, 1992), and appeared independent of skeletal muscle metabolism (Colquhoun *et al.*, 1990; Hettiarachchi *et al.*, 1992). The results of these studies led to a number of possibilities to account for the increases in metabolism (reviewed in Chapter 1, Section 1.3.2; Colquhoun & Clark, 1991). A provocative suggestion was that the energy required to constrict the vascular wall contributed a major portion of the increase in metabolism observed (Chapter 1, Section 1.3.2.4).

The present study confirms the earlier work performed in this laboratory, and by others upon infusion of NE into the hindlimb. Infusion of NE stimulated both α_1- and β-AR. The overall effect was the rapid onset of a sustained increase in perfusion pressure which was only slightly increased upon β-AR blockade at doses of NE greater than 50 nM. Neither rauwolscine (data not shown) nor yohimbine had high affinity for the α-AR, and may have been non-selectively binding α_1-ARs at the doses used (Wilson *et al.*, 1991). The apparent absence of an α_2-AR response may be due to a lack of sufficient basal tone using an *in vitro* perfusion technique (McGrath *et al.*, 1990).

In association with vasoconstriction, 1 nM-0.25 μM NE dose-dependently increased hindlimb $\dot{V}O_2$ and lactate release via stimulation of α_1-AR. During prolonged NE infusion and doses above 50 nM, lactate release included a minor contribution by β-AR-stimulated glycogenolysis.

At doses of NE greater than 0.25 μM, vasoconstriction was associated with dose-dependent decreases in $\dot{V}O_2$ and lactate release from the maximal values obtained with 0.1 μM NE. A dose of 2.5 μM NE decreased hindlimb metabolism to values approaching basal despite increases in perfusion pressure above 200 mmHg. The same profile of increased $\dot{V}O_2$ followed by decreases in $\dot{V}O_2$ below basal despite large increases in resistance has been previously reported in constant-flow perfused hindlimbs at 37°C with erythrocytes (Grubb & Folk, 1976) and 25°C without erythrocytes (Côté *et al.*, 1985). It was proposed that the decrease in $\dot{V}O_2$ was due to closure of some capillaries, thereby limiting the surface area for oxygen diffusion

(Grubb & Folk, 1976). At this high, dose of NE, β-AR-stimulated decreases in $\dot{V}O_2$ and increases in lactate release became more apparent, and were consistent with the observed response to isoproterenol in the hindlimb (Hettiarachchi *et al.*, 1992), although isoproterenol stimulated the β-AR vasodilatory and glycogenolytic responses at lower doses. It has previously been reported that 50 nM isoproterenol inhibited the pressure and $\dot{V}O_2$ responses to 5 nM angiotensin II and 15.6 nM NE, and, by analogy, it would be expected that the β-AR stimulation by the high doses of NE would also vasodilate the angiotensin II response. This was not determined due to problems of synergy between NE (α_1-AR)- and angiotensin II-mediated vasoconstriction, an effect previously reported (Prins *et al.*, 1992). Thus in the presence of propranolol the high dose NE response on perfusion pressure and $\dot{V}O_2$ were greater, suggesting the inhibition of $\dot{V}O_2$ by NE involved both α_1- and β-AR stimulation.

The effects of 5-HT in the hindlimb were not the same as those of NE. 5-HT (50 nM-0.75 μM) induced a dose-dependent increase in perfusion pressure, but surprisingly inhibited $\dot{V}O_2$ uptake to levels well below basal. Both oxygen and pressure effects in the present study were totally blocked by LY 53,857 and ketanserin, but not prazosin, indicating that 5-HT-mediated vasoconstriction occurred via 5-HT_2 (more specifically 5-HT_{2A})-receptors in the hindlimb, a phenomenon also reported in the dog hindlimb (Blackshear *et al.*, 1985), rat jugular vein and aorta (Cohen *et al.*, 1983), rabbit mesenteric artery (Cain & Nicholson, 1989), and to a general extent in most blood vessels including rabbit femoral and tibial arteries (van Nueten, 1985). The vasoconstrictor effects in the rat cremaster muscle have been reported to be due to stimulation of 5-HT_{2A} or 5-HT_{2C} receptors (Alsip & Harris, 1991).

A further disparity between the effects of 5-HT and NE was that 5-HT decreased lactate production to levels slightly below basal during the infusion period, and upon removal, a washout of lactate was observed. The changes in both lactate release and $\dot{V}O_2$ observed upon 5-HT infusion and removal are very similar to those obtained upon reversible occlusion of the hindlimb below the knee, and remain consistent with the observed association between $\dot{V}O_2$ and lactate release (Hettiarachchi *et al.*, 1992). Thus the decrease in lactate release observed during 5-HT infusion may be due to a decrease in the perfused region, although the percent decrease from basal was markedly less than that observed in $\dot{V}O_2$. This may reflect the metabolic fuels used during vasoconstriction, 5-HT tending to utilize anaerobic

metabolism thus producing lactate (see Chapter 3). The washout of lactate upon removal of 5-HT may indicate increased flow to previously underperfused regions. This explanation would depend on 5-HT causing selective constriction of major arteries or arterioles to decrease flow to some regions of the hindlimb. If this were so a shunting mechanism in the vascular system may be involved to allow the constant flow.

Part of the explanation for the decreased metabolism also lies in the direct decrease in $\dot{V}O_2$ mediated by 5-HT in the absence of vasoconstriction. In the presence of the 5-HT_2 antagonist LY 53,857 (10 nM), 0.25 μM 5-HT decreased $\dot{V}O_2$ by 0.5 ± 0.1 $\mu mol.h^{-1}.g^{-1}$ without increasing perfusion pressure (n = 2). This decrease is approx. 30% of the inhibition of $\dot{V}O_2$ observed in the absence of LY 53,857 and could potentially be directly attributed to the vasodilatory action of 5-HT. Unfortunately no direct orphan 5-HT-receptor antagonists were available, so the contributions of vasodilator and constrictor components could not be fully determined. Alsip and Harris (1991) reported that the vasodilatory action of 5-HT was due to stimulation of receptors on smaller arterioles, and is consistent with the dilation observed against angiotensin II. 5-HT appeared to have higher affinity for the dilatory receptor, as dilatation was observed at a 4-fold lower dose than constriction. However, since the overall effect was vasoconstriction, the receptors may be located at different sites within the arterial tree, 5-HT_{2A}-receptors at sites upstream from orphan 5-HT-receptors (Alsip & Harris, 1991). When larger rats were perfused at higher constant-flow rates, thereby increasing basal tone, the dilatory effect of 5-HT was observed before the constriction effect (data not shown). In addition, recent data from constant-pressure perfused hindlimbs (Ji-Ming Ye, personal communication) has shown 5-HT mediated a large increase in flow at doses below 0.1 μM, suggesting stimulation of orphan 5-HT-receptors, and at higher doses decreased flow rate, presumably due to stimulation of 5-HT_{2A} receptors.

The observed washout of a small number of erythrocytes upon infusion of NE, but not 5-HT tends to suggest NE was causing a small portion of the flow to reach previously underperfused regions, whereas 5-HT was not.

The difference in metabolic effects could be attributed to the possibility that the two agonists act at different regions of the vascular tree. This view is supported by studies showing the location of adrenergic nerve terminals and receptors, which appear to increase in number surrounding the small arteries and arterioles (Fuxe & Sedvall, 1965; Nilsson *et al.*, 1986; Hirst & Edwards, 1989). Also, the sensitivity to

abluminally applied NE decreases with increasing arteriolar size (Gray, 1971). In contrast, 5-HT was reported to vasoconstrict larger arteries, and not small arteries or arterioles (Hollenberg, 1985; Blackshear *et al.*, 1985; Alsip & Harris, 1991), and dilate smaller arterioles (Alsip & Harris, 1991). A study performed by Lamping *et al.* (1989) demonstrated that in the feline coronary microcirculation 5-HT stimulated constriction in arteries and arterioles larger than 90 μm but dilated arterioles smaller than 90 μm, and vasopressin (another thermogenic vasoconstrictor in the hindlimb, Colquhoun *et al.*, 1988) constricted arterioles less than 90 μm but had no effect on larger arteries. In addition the total inhibition of the NE response by 50 nM isoproterenol (Colquhoun *et al.*, 1990) may also suggest the vasoconstriction was occurring on smaller vessels, as β-AR were proposed to be located on small resistance vessels and precapillary sphincters (Lundvall & Hillman, 1978; Lundvall *et al.*, 1982). For the same reasons, the lack of inhibition of the 5-HT response supports the proposal that 5-HT_{2A} receptors are located on larger vessels.

The effects of 5-HT in a less complex vascular system, the isolated perfused mesenteric artery arcade, were then determined. In the absence of vascular tone the $\dot{V}O_2$ by the preparation was minimal, suggesting a lack of contribution to metabolism by surrounding lymph and fat. This contrasts with the maximally dilated hindlimb where there is significant oxygen consumption (Ye *et al.*, 1990b; Colquhoun *et al.*, 1990), presumably due to skeletal muscle metabolism. Prior experiments involving the perfusion of isolated rat mesenteric artery network by Ye *et al.* (1990a) showed that NE induced an increase in perfusion pressure and oxygen uptake, consistent with the vascular contribution to vasoconstrictor-induced increases in metabolism. The existence of 5-HT_{2A} receptors in isolated rabbit mesenteric artery was reported by Cain and Nicholson (1989) who showed that 5-HT-induced constriction in the range 0.3-3.0 μM was inhibited by 30 nM ketanserin whereas 0.1 μM prazosin had no effect. In the present study with rat mesenteric artery it was found that 5-HT induced dose-dependent vasoconstriction with an associated increase in $\dot{V}O_2$ and lactate release via stimulation of 5-HT_{2A} receptors. These results showed that for a less complex vascular system 5-HT-stimulated vasoconstriction was associated with an increase in $\dot{V}O_2$, thereby supporting the possibility that the observations in the hindlimb were not due to a direct 5-HT-mediated inhibition of smooth muscle metabolism.

These results, in conjunction with literature reports, suggest NE and 5-HT contribute to the control of hindlimb metabolism by site-selective vasoconstriction.

Lower doses of NE stimulated α_1-AR probably located on arterioles within the microcirculation, increased vascular smooth muscle metabolism, and may also have redistributed flow to highly metabolically active regions. Conversely, 5-HT_2-receptors and possibly a separate subgroup of α_1-AR are proposed to be located at discrete vascular sites on larger blood vessels that, when stimulated, selectively redistributed blood-flow (functional vascular shunting), resulting in reduction of oxygen and glucose delivery to metabolically active hindlimb tissues.

CHAPTER 3

Metabolic characteristics of vasoconstriction.

3.1 Introduction.

The contrasting effects of norepinephrine (NE) and serotonin (5-HT) on oxygen uptake ($\dot{V}O_2$) in association with vasoconstriction in the constant-flow perfused rat hindlimb described in Chapter 2 suggest that metabolism may be controlled in the resting state by alterations in flow distribution. The different anatomical location of hormone receptors may have important implications *in vivo*. During systemic hypoxia, flow is diverted away from nutritive capillaries without compromising tissue need (Harrison *et al.*, 1990b) by altering the locus of myogenic tone to larger blood vessels (Bertuglia *et al.*, 1991), predominantly by increased sympathetic nerve activity (Mian & Marshall, 1991a). In general, larger vessels are usually able to maintain constriction during hypoxic conditions (Shibata & Briggs, 1967; Altura & Altura, 1976; De Mey & Vanhoutte, 1983). Conversely, small blood vessels tend to dilate in response to low oxygen tensions (Carrier *et al.*, 1964; Duling, 1972; Harris *et al.*, 1976). This may be due to local regulatory processes or an increase in dependency on oxidative metabolism with decreasing vessel size.

This chapter focuses on the metabolic characteristics of vasoconstriction by NE and 5-HT. As 5-HT and high doses of NE are proposed to interact at sites on larger vessels (Blackshear *et al.*, 1985; Alsip & Harris, 1991; Tuncer *et al.*, 1992) than low doses of NE (Gray, 1971; Hirst & Edwards, 1989; Lamping *et al.*, 1989), the possibility that the sites may be distinguished on the basis of their oxidative and glycolytic capacity was examined. To assess oxygen-dependency, the effects of vasoconstrictors were examined when coupled mitochondrial oxidative phosphorylation was either interrupted or placed under hypoxic conditions. In addition, carbohydrate-dependency of vasoconstriction was determined by omitting glucose from the perfusion medium.

3.2 Materials and methods.

3.2.1 Perfused hindlimbs.

Surgery was performed on 180-200 g rats as outlined in Section 2.2.1.2.1. Experiments were performed at 25°C and 4.0 ± 0.1 $ml.min^{-1}$ with 2% bovine serum albumin (BSA) added to the perfusion medium. Additional details are given in Section 2.2.1. During hypoxic perfusions 95% N_2-5% CO_2 replaced 95% O_2-5% CO_2.

To avoid organic solvent perturbation of the system, a water soluble mitochondrial uncoupler, sodium azide was used.

3.2.2 Statistical analysis.

The statistical significance of differences between groups of data were assessed by the unpaired, two-tailed Student's *t* test. Significant differences were recognized at $P < 0.05$.

3.3 Results.

3.3.1 Effects of vasoconstrictors.

Under fully aerobic basal conditions, mean arterial P_{O_2} was 697.5 ± 4.8 mmHg ($n = 23$), mean venous P_{O_2} was 425.2 ± 7.4 mmHg ($n = 23$) with a $\dot{V}_{O_2}$ of 6.6 ± 0.1 $\mu mol.h^{-1}.g^{-1}$, and the perfusion pressure was 23.1 ± 0.6 mmHg ($n = 23$, Table 3.1). These parameters remained constant for at least 3 h if no additions were made. Infusion of 50 nM NE and 0.25 μM 5-HT produced comparable increases in perfusion pressure of approximately 100% above basal. In association with the rise in pressure, 50 nM NE induced an increase in $\dot{V}_{O_2}$ of 57.3 ± 5.1 % ($n = 6$), whereas 5-HT decreased $\dot{V}_{O_2}$ from basal by 24.8 ± 4.4 % ($n = 8$, Table 3.2). NE-induced effects approached steady state within 5 min of infusion and remained constant provided NE was not withdrawn. 5-HT effects were different from those of NE and, although showing relatively rapid onset, continued to increase gradually throughout the period of infusion. Venous P_{O_2} and perfusion pressure values were taken 15 min after agonist infusion. Basal conditions were achieved rapidly upon removal of either

Inhibitor	*n*	P_aO_2 (mmHg)	P_vO_2 (mmHg)	$\dot{V}O_2$ ($\mu mol.h^{-1}.g^{-1}$)	Pressure (mmHg)
No Inhibitor	36	697.5 ± 4.8	425.2 ± 7.4	6.6 ± 0.1	23.1 ± 0.6
N_2-CO_2	12	27.8 ± 2.7	2.8 ± 0.4	0.6 ± 0.1	21.9 ± 0.7
Cyanide (1 mM)	10	699.5 ± 8.3	658.5 ± 12.3	1.0 ± 0.2	22.7 ± 1.3
Azide (1 mM)	8	684.2 ± 14.4	220.0 ± 15.9	11.0 ± 0.4	22.0 ± 1.6

Table 3.1 Effects of hypoxia, cyanide, and azide on hindlimb $\dot{V}O_2$ and perfusion pressure under basal conditions.

Values are means ± SE. *n*, no. of hindlimb perfusions; P_aO_2, arterial PO_2; P_vO_2, venous PO_2; $\dot{V}O_2$, oxygen uptake. A gas mixture of 95% N_2-5% CO_2 was used to induce hypoxia.

Inhibitor	50 nM NE			0.25 μM 5-HT		
	$\dot{V}O_2$ ($\mu mol.h^{-1}.g^{-1}$)	Pressure (mmHg)	*n*	$\dot{V}O_2$ ($\mu mol.h^{-1}.g^{-1}$)	Pressure (mmHg)	*n*
No Inhibitor	10.1 ± 0.3 (57.3 ± 5.1%)	41.3 ± 2.0 (97.5 ± 10.5%)	6	4.6 ± 0.3 (-24.8 ± 4.4%)	46.9 ± 3.3 (145.9 ± 22.5%)	8
N_2-CO_2	0.8 ± 0.1 (0%)	25.1 ± 0.7 (4.3 ± 0.4%)	7	0.6 ± 0.1 (0%)	51.1 ± 6.7 (136.5 ± 25.2%)	7
Cyanide (1 mM)	0.9 ± 0.2 (0%)	25.3 ± 1.9 (7.2 ± 3.1%)	6	0.8 ± 0.2 (0%)	49.2 ± 3.7 (129.2 ± 32.3%)	6
Azide (1 mM)	11.5 ± 0.3 (4.0 ± 1.1%)	24.8 ± 1.8 (3.6 ± 1.4%)	5	7.7 ± 0.4 (-30.0 ± 2.5%)	58.0 ± 9.2 (139.5 ± 22.6%)	5

Table 3.2 Effects of NE and 5-HT on hindlimb $\dot{V}O_2$ and perfusion pressure during hypoxia and in the presence of cyanide or azide.

Values are means ± SE, with percentage change from basal given in parentheses. Where appropriate, values were taken 15 min after commencement of agonist. Basal $\dot{V}O_2$ and perfusion pressure values are given in Table 3.1. A gas mixture of 95% N_2-5% CO_2 was used to induce hypoxia. NE, norepinephrine; 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake; *n*, no. of hindlimb perfusions.

agonist (Figure 3.1).

3.3.2 Oxygen-dependence.

The effects of hypoxia, cyanide, and azide on hindlimb $\dot{V}O_2$ and perfusion pressure under basal conditions are summarized in Table 3.1. Infusion of potassium cyanide (1 mM) almost totally inhibited $\dot{V}O_2$, and sodium azide (1 mM) led to an increase in $\dot{V}O_2$ consistent with partial uncoupling of oxidative phosphorylation. Gassing with 95% N_2, 5% CO_2 decreased PO_2 levels almost to zero. No significant effect on basal perfusion pressure was observed for any of these treatments.

Table 3.2 shows the effects of hypoxia, cyanide, and azide on NE- and 5-HT-mediated changes in pressure and $\dot{V}O_2$. Whereas the vasoconstrictor effect of 50 nM NE were almost totally opposed (approximately 95%) by each of these treatments, 0.25 μM 5-HT was able to maintain constriction without being significantly affected (Table 3.2). Addition of agonists in the presence of cyanide or hypoxia had no effect on $\dot{V}O_2$. In the presence of azide, NE increased $\dot{V}O_2$ by only a marginal amount (4.0 ± 1.1 %; n = 5) and 5-HT decreased $\dot{V}O_2$ by 30.0 ± 2.5 % (n = 5) from 11.0 ± 0.3 to 7.7 ± 0.4 $\mu mol.h^{-1}.g^{-1}$, an effect of approximately the same proportion as that seen when azide was absent.

3.3.2.1 Dose-dependent effects of NE.

Hypoxia, cyanide and azide abolished NE-mediated effects at concentrations below 50 nM. At higher concentrations (50 nM-1 μM), a dose-dependent increase in pressure occurred (Figure 3.2). Under hypoxic conditions the constriction induced by 1 μM NE was markedly inhibited (76 ± 3%, n = 5) by ketanserin (0.1 μM) (data not shown) and almost totally inhibited by prazosin (50 nM) (91 ± 2%, n = 3; Figure 3.3). Similar results were obtained in the presence of cyanide (data not shown).

When hindlimbs were perfused with medium equilibrated against 95% O_2-5% CO_2, the vasoconstriction induced by 50 nM NE and by 0.25 μM 5-HT was inhibited by 14 ± 4% (n = 3) and 100% (n = 3) respectively upon infusion of 0.1 μM ketanserin. Addition of 25 nM prazosin totally blocked all NE-induced responses but presumably had no effect on 5-HT-mediated vasoconstriction at this low dose (1 μM prazosin had no effect, Chapter 2, Figure 2.5).

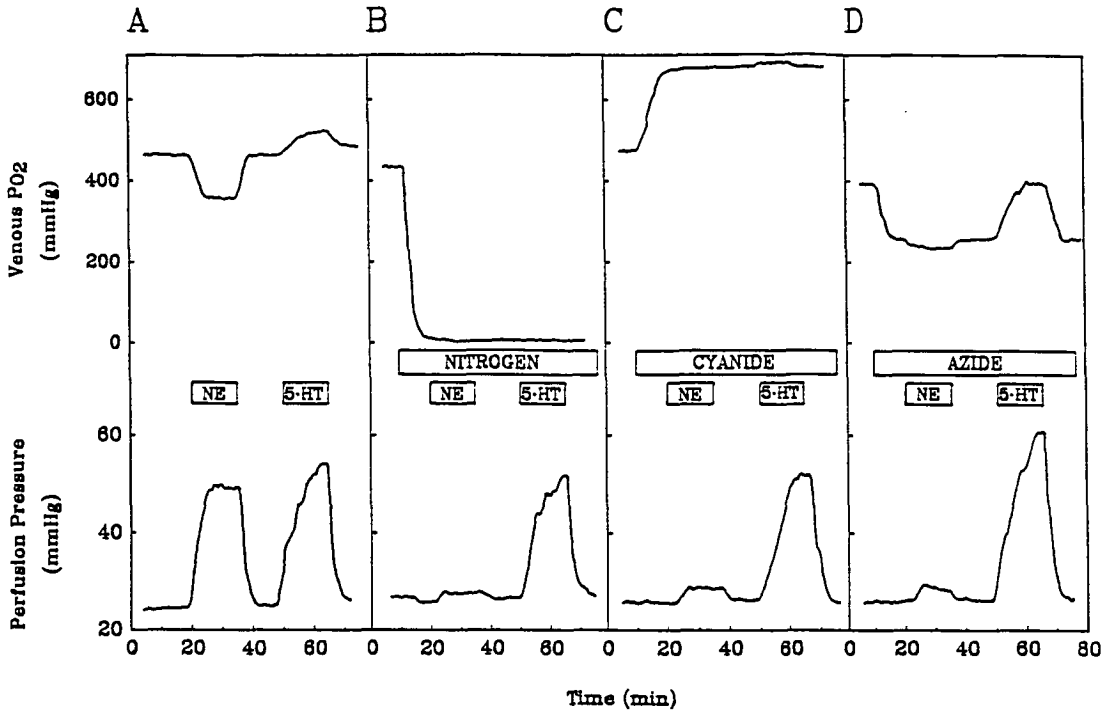

Figure 3.1 Effects of hypoxia, cyanide, and azide on NE- and 5-HT-mediated changes in hindlimb venous PO_2 and perfusion pressure.

Hindlimbs from 180-200 g rats were perfused at 25°C and a constant flow rate of 4 $ml.min^{-1}$ with perfusion medium containing 2% bovine serum albumin. 50 nM NE and 0.25 μM 5-HT were infused for 15 min under each condition. Perfusion medium, initially equilibrated against 95% O_2-5% CO_2, was either maintained (*A*, *C*, and *D*) or switched to one equilibrated against 95% N_2-5% CO_2 (*B*), as shown. Other additions were 1 mM potassium cyanide (*C*) or 1 mM sodium azide (*D*). Representative traces are shown, and mean values from all experiments are given in Table 3.2. NE, norepinephrine; 5-HT, serotonin. From Dora *et al.* (1992).

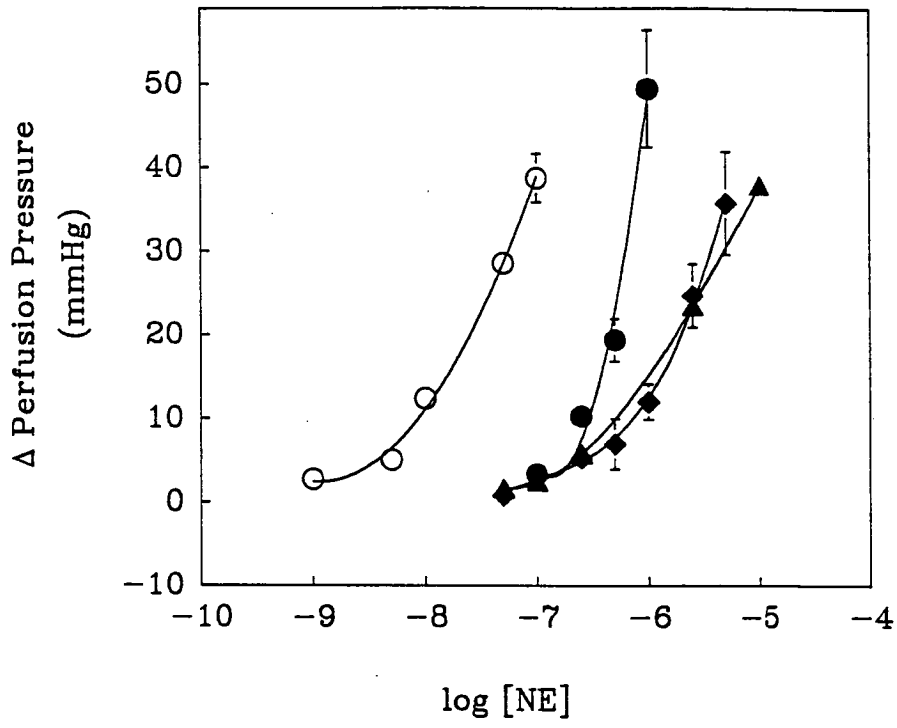

Figure 3.2 Effect of hypoxia, cyanide and azide on dose-response curve for NE on hindlimb perfusion pressure.

Hindlimbs from 180-200 g rats were perfused at 25°C and a constant flow rate of 4 $ml.min^{-1}$ with perfusion medium containing 2% bovine serum albumin. Perfusion media for hindlimbs were equilibrated against 95% N_2-5% CO_2 (●); or against 95% O_2-5% CO_2 in the absence (O) or presence of 1 mM potassium cyanide (▲) or 1 mM sodium azide (◆). For each treatment, basal perfusion pressures and oxygen tensions are given in Table 3.1. Values are means ± SE for a minimum of 3 perfusions; when not visible, error bars are within symbol. NE, norepinephrine.

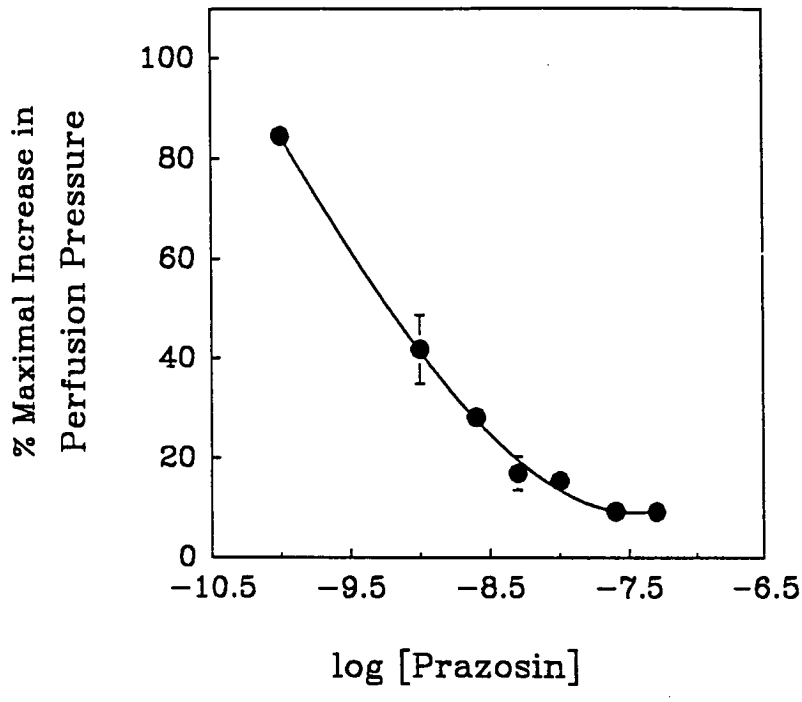

Figure 3.3 Dose-response curves for prazosin against NE on hindlimb perfusion pressure under hypoxic conditions.

Hindlimbs from 180-200 g rats were perfused at 25°C and a constant flow rate of 4 $ml.min^{-1}$ with perfusion medium containing 2% bovine serum albumin and equilibrated against 95% N_2-5% CO_2. Mean basal perfusion pressure was 20.3 ± 0.3 mmHg, and oxygen tensions were 24.3 ± 6.2 mmHg (arterial) and 0.5 ± 0.4 mmHg (venous). Values are means ± SE for 3 hindlimbs; when not visible, error bars are within symbols. Prazosin was infused in the presence of 1 μM NE. NE, norepinephrine.

3.3.3 Carbohydrate-dependence.

The presence of glycogen stores in both skeletal and smooth muscle cells complicated the determination of carbohydrate-dependence of agonist-induced vasoconstriction. It was considered that elimination of glucose from the perfusion medium and exposure to cyanide would markedly deplete smooth muscle glycogen stores in a manner analogous to Furchgott (1966), who found glycogen stores of rabbit aorta were largely depleted by exposure to anoxia in the absence of glucose for about 1 hour. Under these conditions the transient nature of vasoconstriction by 5-HT suggested that at least those smooth muscle cells with 5-HT_2 receptors became glycogen-depleted and thus carbohydrate-free. The reversible nature of cyanide-induced inhibition of $\dot{V}O_2$ then allowed determination of the carbohydrate-dependence of vasoconstriction.

Removal of glucose from the perfusion medium had no significant effect on basal hindlimb $\dot{V}O_2$, lactate release or perfusion pressure. In addition the responses to 50 nM NE and 0.25 μM 5-HT for the 15-min period of infusion were not affected (data not shown). However, when cyanide (1 mM) was present and glucose was omitted, it was noted that 5-HT (0.25 μM) was no longer able to mediate a constant vasoconstrictive effect; rather, a transient increase in perfusion pressure was followed by a gradual return to basal pressure (Figure 3.4). During the period of 5-HT-mediated increased perfusion pressure in the presence of cyanide, hindlimb lactate efflux increased markedly from a basal value of 10.8 ± 0.3 to a peak value of 24.3 ± 1.6 $\mu mol.h^{-1}.g^{-1}$ corresponding to the peak in perfusion pressure, and then decreased to basal within 2 min. The average pressure peak for 5-HT in the presence of cyanide and the absence of glucose was 61.1 ± 3.5 mmHg, representing an increase of 28.8 ± 2.5 mmHg (n = 4). The period during which constriction could be maintained during these conditions was surprisingly constant (24.5 ± 2.5 min; n = 4). In some of these experiments, muscle rigor became apparent and pressure steadily increased despite removal of agonist; thus cessation of constriction was less readily discernible.

On those occasions when muscle rigor was not observed, it was found that the cyanide effects on $\dot{V}O_2$ were reversible (Figure 3.4). 30 min after cyanide removal, $\dot{V}O_2$ was 7.0 ± 0.4 $\mu mol.h^{-1}.g^{-1}$ (n = 6). Under these conditions and still in the absence of glucose, 0.25 μM 5-HT-mediated effects were of the same magnitude as that observed when glucose was present (Figure 3.5). However, the effects of 50 nM NE on perfusion pressure and lactate efflux were significantly reduced (Figure

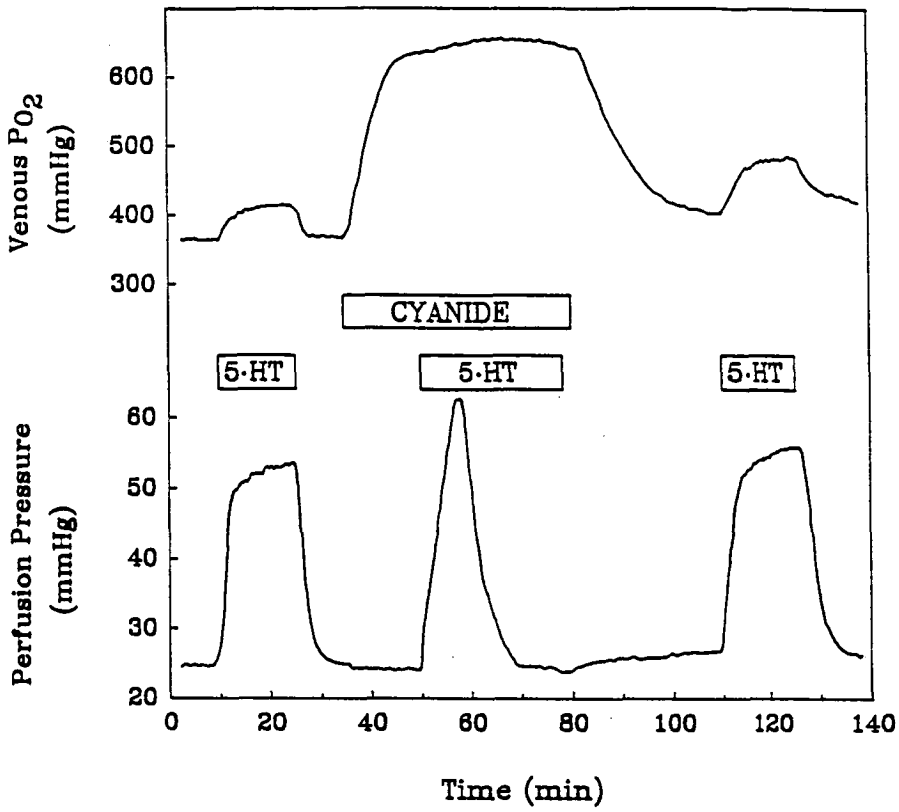

Figure 3.4 **Effect of glucose-free perfusion medium on the hindlimb response to a representative dose of 5-HT in the absence and presence of cyanide.**

Representative traces for venous P_{O_2} and perfusion pressure are given for hindlimbs (n = 3) perfused at 4 ml.min^{-1} with glucose-free medium equilibrated against 95% O_2-5% CO_2. 5-HT (0.25 μM) was infused before, during and after treatment with potassium cyanide (1 mM).

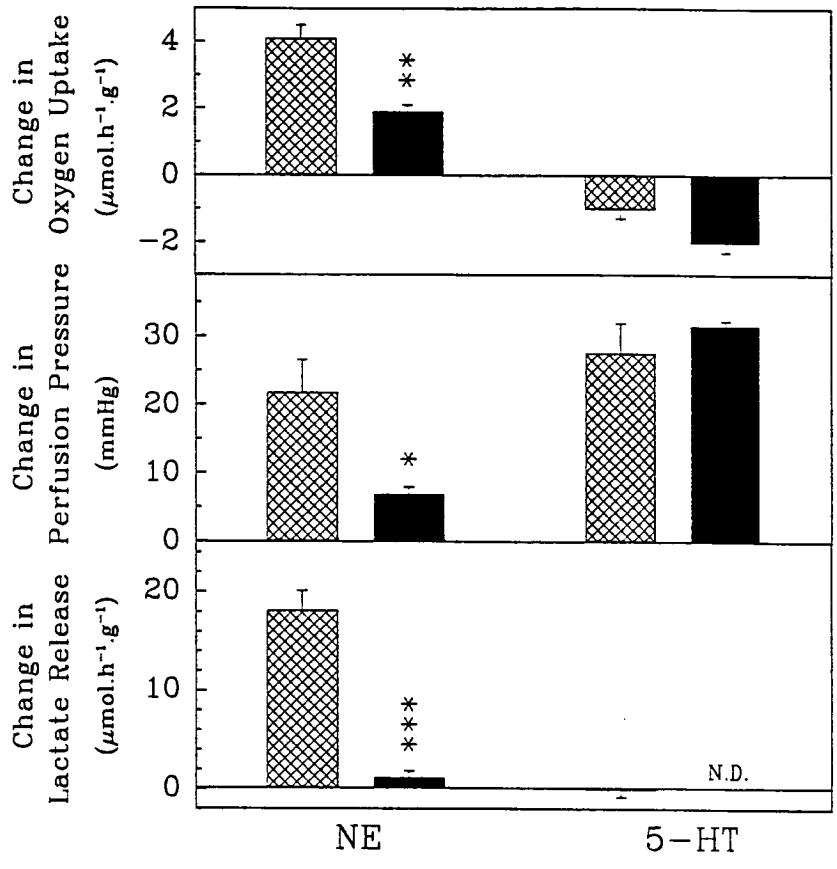

Figure 3.5 Carbohydrate-dependence of the hindlimb response to NE and 5-HT.

Hindlimbs from 180-200 g rats were perfused at 25°C and a constant flow rate of 4 ml.min^{-1} with perfusion medium containing 2% bovine serum albumin. $\dot{V}O_2$, perfusion pressure and lactate release were monitored in the presence of glucose (▩) and absence of glucose and marked depletion of smooth muscle glycogen stores (■, glycogen-depletion method described in Section 3.3.3). Mean basal $\dot{V}O_2$, lactate release and perfusion pressure were 6.7 ± 0.4 μmol.h^{-1}.g^{-1}, 7.8 ± 0.8 μmol.h^{-1}.g^{-1} and 21.6 ± 0.6 mmHg (n = 6) respectively in the presence of glucose, and 7.0 ± 0.4 μmol.h^{-1}.g^{-1}, 7.4 ± 0.5 μmol.h^{-1}.g^{-1} and 23.5 ± 0.8 mmHg (n = 6) respectively when carbohydrate was markedly reduced. Values obtained after 10 min of 50 nM NE or 0.25 μM 5-HT infusion (n = 3) are expressed as means ± SE. N.D., not determined; NE, norepinephrine; 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

3.5). Subsequent reintroduction of glucose to the medium increased the NE response towards that observed in the presence of oxygen and glucose (change in perfusion pressure of 16.5 ± 1.5 mmHg, $n = 2$).

3.4 Discussion.

The main observation from this study was that hindlimb vasoconstriction induced by the agonists NE and 5-HT was affected differently when either coupled oxidative phosphorylation was interrupted or when smooth muscle carbohydrate levels were markedly reduced. Whereas 0.25 μM 5-HT-induced vasoconstriction was not significantly affected by hypoxia, addition of cyanide, azide, or reduction of available carbohydrate, vasoconstriction produced by 50 nM NE was almost totally opposed by these perturbations. In addition, at higher doses of NE, 5-HT-like effects were observed.

Overall, these findings suggest that there is a biochemical heterogeneity of smooth muscle in vessels supplying hindlimb tissue. Vascular smooth muscle stimulated to constrict by 5-HT or high doses of NE, appears capable of either aerobic or anaerobic metabolism, depending on the supply of fuels, and becomes dependent on exogenous glucose to maintain energy requirements for vasoconstriction when glycogen reserves presumably become exhausted and oxidative phosphorylation is inhibited. In contrast, vessels stimulated by lower doses of NE appear to be totally dependent on oxidative metabolism and are also partially dependent on glycolytic activity. In general, the data do not appear to be consistent with the proposal (Paul *et al.*, 1979) that metabolism in vascular smooth muscle (coronary artery) is functionally compartmentalized, with suprabasal oxidative metabolism associated primarily with the energy requirements of the actin-myosin ATPase and aerobic glycolysis linked to the energy requirements of membrane-related processes. This may, of course, be related to the size of vessel Paul *et al.* (1979) used in their studies.

The differential ability of various smooth muscle preparations to respond to vasoconstricting agents under hypoxic conditions has been observed previously. The major determinants appear to be anatomical location and vessel wall thickness. For example, most (Altura & Altura, 1976; De Mey & Vanhoutte, 1983; Shibata & Briggs, 1967) but not all (Coburn *et al.*, 1979; Pittman & Duling, 1973) early studies on larger arterial segments demonstrated that high doses of both epinephrine and NE

could induce vasoconstriction under hypoxic and anoxic conditions. This was shown to be glucose-dependent (Altura & Altura, 1976; De Mey & Vanhoutte, 1983; Shibata & Briggs, 1967). At more physiological doses, both NE and epinephrine constricted to between 10 and 30% of the normoxic response, and there was a dose curve shift to the right (Detar & Bohr, 1968; Hellstrand *et al.*, 1977). Anoxic conditions have been reported to either augment (van Nueten & Vanhoutte, 1980) or attenuate (Nair & Dyer, 1972; Schmidt *et al.*, 1990) 5-HT-induced vasoconstriction.

Similarly, when oxygen was present but carbohydrate reduced, generally only slightly attenuated (Furchgott, 1966; Coe *et al.*, 1968), although sometimes markedly attenuated (Altura & Altura, 1970; Adams & Dillon, 1989) NE- or epinephrine-induced vasoconstriction was observed. In the present study, the ability of NE to stimulate vasoconstriction in the absence of glucose in the perfusion medium, but not when the glycogen stores were partially depleted, is consistent with studies showing glycogen was utilized as the primary oxidative substrate during active tension generation (Lynch & Paul, 1983, 1987), and activity of glycogen debrancher enzyme limited glycogen utilization (Lynch *et al.*, 1989) in partially glycogen-depleted NE-stimulated vessels. However, the ability of 5-HT to maintain vasoconstriction even in carbohydrate-depleted conditions is consistent with the findings of Lynch *et al.* (1989) with KCl in the carotid artery. Thus the data presented here provides further evidence supporting the heterogeneity of blood vessel metabolism.

Glycolytic capacity is also determined by vessel wall thickness. Diffusion of oxygen through the layers of smooth muscle is predicted to decrease with increases in number of layers (Pittman & Duling, 1973), and, depending on the density and distribution of vasa vasorum, the more distal layers may need larger supplies of anaerobic substrates such as glycogen to maintain energy requirements. In general, smaller vessels dilate more readily in response to low oxygen concentrations than larger arteries (Carrier *et al.*, 1964; Duling, 1972; Harris, 1976). This may imply that the metabolic requirement for oxygen is more acutely developed and that glycogen stores are lower in smaller vessels, which are unable to hold pressure unless oxygen is present. Alternatively, hypoxia may have no direct dilating effect on small vessels but rather may cause an imbalance between local relaxing and constricting factors.

The apparent difference in oxygen and carbohydrate dependency between sites of constriction may suggest that NE normally acts at sites further down the arterial tree than does 5-HT. Work by others has found that 5-HT generally constricts larger arteries (Blackshear *et al.*, 1985; Hollenberg, 1989; Lamping *et al.*,

1989; Alsip & Harris, 1991) and the sensitivity to NE, at least on the abluminal surface of vessels, decreases with increasing arteriolar size (Gray, 1971). Increases in pressure due to low doses of NE are associated with increased $\dot{V}O_2$ (Grubb & Folk, 1976; Richter *et al.*, 1982; Côté *et al.*, 1985; Colquhoun *et al.*, 1988) and are blocked by nitroprusside (Colquhoun *et al.*, 1990) and prazosin (see Section 2.3.1), and therefore the sites are likely to be α_1-adrenergic and located on smaller vessels. At high doses of NE, vasoconstriction appears to be mediated at another group of adrenergic sites that also are blocked by prazosin. These represent a subgroup of α_1-adrenoceptor (AR) sites that have a relatively low affinity for NE, a high affinity for ketanserin, and, like 5-HT sites, are located on larger vessels. Alternatively, the receptors stimulated by only high doses of NE may be located abluminally, the endothelial cell layer of arterioles forming a diffusion barrier (Lew *et al.*, 1989).

5-HT-mediated vasoconstriction appears to result in an inhibition of hindlimb $\dot{V}O_2$ due to shunting of perfusate away from microvascular elements involved in thermogenesis (Dora *et al.*, 1991) without opening direct arteriovenous shunts (see Section 7.3.3). Supporting evidence that 5-HT acts at sites on larger arteries to induce vascular shunting of flow also comes from experiments in this study in which azide was used to uncouple, in part, oxidative phosphorylation. $\dot{V}O_2$ was increased by azide, but 5-HT-induced vasoconstriction was not significantly different from control, and the percentage decrease in $\dot{V}O_2$ due to 5-HT action was similar in magnitude to that when 5-HT was used alone (Table 3.2).

The ability to induce site-selective vasoconstriction may play an important physiological role. For example, systemic hypoxia has been shown to increase blood flow to canine femoral artery dramatically, although flow was diverted through high-flow non-nutritive shunts, resulting in a reduction in overall capillary flow but maintenance of tissue oxygenation (Harrison *et al.*, 1990b). In rat spinotrapezius muscle, the locus of vasomotion during systemic hypoxia changes from the small arterioles (A4) to the transverse arterioles (A3, Bertuglia *et al.*, 1991; see Chapter 8, Figure 8.1 for arterial branching nomenclature). The increase in tone has been predominantly attributed to sympathetic nerve α_1-AR stimulation (Mian & Marshall, 1991b), suggesting a role for the increase in nerve density at the transverse arteriole roots (Saltzman *et al.*, 1992). Another function for site-selective vasoconstriction might be in situations of trauma, in which involvement of 5-HT is likely but not established, and acute haemorrhage (reviewed by Lewis & Post, 1982). However,

under these conditions, plasma levels of 5-HT are lower than normal, probably the result of uptake by circulating platelets and endothelial cells, particularly in the pulmonary artery (Bhat & Block, 1990). If a role for 5-HT-mediated site-selective vasoconstriction exists, it should be important to note that synergism between 5-HT and NE is likely because low levels of 5-HT can amplify the vasoconstrictor effects of NE, such as in the femoral artery (van Nueten & Janssens, 1989).

CHAPTER 4

Sub-classification of α_1-AR- and 5-HT-receptor stimulation.

4.1 Introduction.

Infusion of norepinephrine (NE) into the constant-flow perfused rat hindlimb at 25°C stimulated an increase in oxygen uptake ($\dot{V}O_2$) and lactate release in association with vasoconstriction over the dose range 1-250 nM (Chapter 3). At higher doses the increases in metabolism were inhibited to levels below basal despite further rises in pressure. The responses to NE were totally blocked by the α_1-adrenoceptor (AR) antagonist prazosin, and only slightly augmented by the β-AR antagonist propranolol (Section 2.3.1).

The ability of NE to stimulate prazosin-sensitive responses that were independent of oxidative-metabolism at high doses, but not at low doses, suggested the presence of different α_1-AR subtypes in the hindlimb (Dora *et al.*, 1992, Chapter 3). Thus since it was possible that two subgroups of α_1-AR were controlling hindlimb metabolism by opposing mechanisms, it was considered of interest to further characterize the subgroups involved. Recently, rat α_1-ARs have been classified into subtypes according to their extracellular Ca^{2+}-dependence during vasoconstriction (Han *et al.*, 1987), and by use of selective α_1-AR antagonists (Han & Minneman, 1991; Ruffolo *et al.*, 1991).

α_{1A}-AR stimulation is coupled to a pertussis toxin-sensitive G-protein (Gilman, 1987) which causes an influx of Ca^{2+} through receptor-operated Ca^{2+}-channels (Figure 4.1). In various preparations it has been observed that 5-methyl urapidil (5-MU) has a high affinity for α_{1A}-ARs (Han & Minneman, 1991).

α_{1B}-AR stimulation is coupled to a pertussis toxin-insensitive G-protein (Gilman, 1987). Subsequent activation of phospholipase C and formation of inositol triphosphate (IP_3) signals the mobilization of intracellularly-bound calcium (Berridge & Irvine, 1984; Figure 4.1). Hence α_{1B}-AR stimulated constriction was independent of extracellular Ca^{2+} (Han *et al.*, 1987). Chloroethylclonidine (CEC) has high affinity for α_{1B}-ARs (Han & Minneman, 1991). There is growing pharmacological and structural evidence, however, for intra- and inter-species subdivision of ARs (reviewed in Ruffolo *et al.*, 1991).

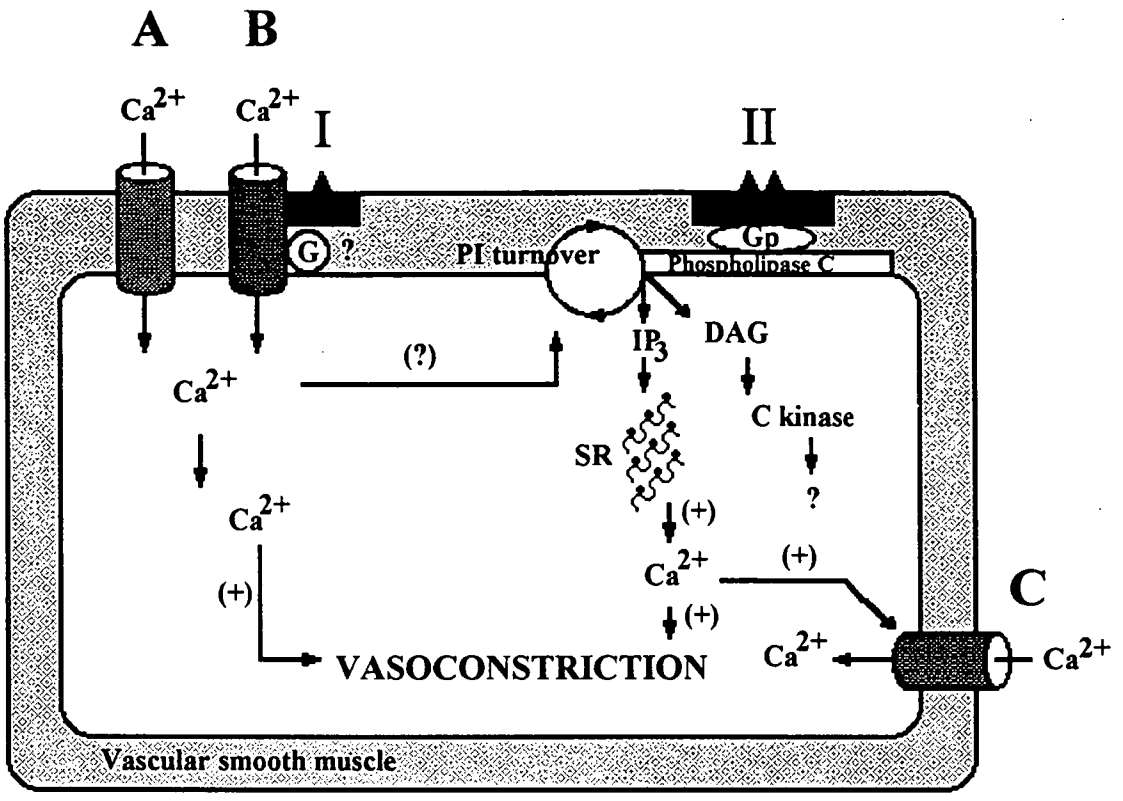

Figure 4.1 Receptor-mediated events leading to vasoconstriction in vascular smooth muscle.

I: α_{1A}-AR coupled to a pertussis toxin sensitive G-protein with high affinity for low doses of NE, prazosin and 5-MU.

II: α_{1B}-AR or 5-HT_{2A}-receptor coupled to a pertussis toxin insensitive G-protein with high affinity for either high doses of NE and CEC or 5-HT and LY 53,857, respectively.

A: Voltage-gated Ca^{2+}-channel stimulated by 5-HT_1-like receptor agonists.

B: Receptor-operated Ca^{2+}-channel.

C: Ca^{2+}-channel stimulated by rises in intracellular Ca^{2+}.

AR, adrenoceptor; NE, norepinephrine; 5-HT, serotonin; 5-MU, 5-methyl urapidil; CEC, chloroethylclonidine; IP_3, inositol 1,4,5-triphosphate; DAG, 1,2-diacylglycerol; G, G-protein; SR, sarcoplasmic reticulum.

Modified and extended from Suzuki *et al.* (1990).

Smooth muscle 5-HT_{2A} receptor stimulation has been linked to generation of IP_3 and subsequent Ca^{2+} release from internal stores (Doyle *et al.*, 1986) in a manner similar to α_{1B}-AR-mediated release. Recently the constrictor response to 5-HT has been attributed to stimulation of two separate receptors in isolated dog saphenous vein (Sumner *et al.*, 1992). 5-HT_1-like, but not 5-HT_{2A}-, receptor stimulation appears dependent on extracellular Ca^{2+} for vasoconstriction, and thus the two receptor subtypes may be classified according to their extracellular Ca^{2+}-dependence in a manner similar to the α_1-ARs.

In this study, the extracellular Ca^{2+}-dependence of NE- and 5-HT-induced effects were determined in the rat hindlimb. In addition, selective α_1-AR antagonists were employed to further characterize the NE-mediated responses.

4.2 Materials and methods.

4.2.1 Perfused hindlimbs.

Surgery was performed on 180-200 g rats as outlined in Section 2.2.2. Experiments were performed at 25°C and 4.1 ± 0.1 $ml.min^{-1}$ with 2% bovine serum albumin (BSA) added to the perfusion medium. Additional details are given in Sections 2.2.3-2.2.6.

4.2.1.1 Modification of extracellular [Ca^{2+}].

In some experiments the Ca^{2+} concentration was manipulated by adding ethyleneglycol-bis-(b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and/or less Ca^{2+} to the perfusion medium. Perfusions were performed using medium containing 1.27 mM Ca^{2+} and 1.27 mM EGTA, 0.1 mM Ca^{2+}, or no added Ca^{2+} and 0.1 mM EGTA. When equimolar Ca^{2+} and EGTA were added to the perfusion medium at 25°C the free [Ca^{2+}] was 3.0 ± 0.1 μM (n = 4), measured with a Selectrode Ca^{2+} electrode (Denmark) and TPS Programmable Ion Analyser (Australia).

Hindlimbs were allowed to equilibrate with medium containing 1.27 mM Ca^{2+} for 30 min. Perfusion medium with altered Ca^{2+} concentrations were then introduced and due to the resulting transient increase in perfusion pressure (Table 4.1), equilibration under these Ca^{2+}-manipulated conditions continued for at least a further 60 min.

Most perfusions were performed in the presence of 10 μM (±)-propranolol. Antagonists, except CEC, were infused throughout the experiments and were allowed to equilibrate for at least 20 min prior to infusion of NE. CEC is an alkylating agent which interacts in an apparent irreversible fashion. CEC was infused for 20 min three times (10 min between each addition) then removed before NE addition, according to the method described by Suzuki *et al.* (1990). In prior experiments, increasing the repeated infusions of CEC did not further attenuate the response to NE. It was also noted that part of the CEC response was reversible as despite repeated CEC pretreatment, infusion of CEC in the presence of NE slightly (< 10%) attenuated the NE-mediated response. However, to remain consistent with literature methods (Suzuki *et al.*, 1990; Han & Minneman, 1991; Piascik *et al.*, 1991; Oriowo *et al.*, 1992; Oriowo & Ruffolo, 1992), CEC was not infused in the presence of NE.

4.2.2 Statistical analysis.

The statistical significance of differences between groups of data were assessed by the unpaired, two-tailed Student's *t* test. Significant differences were recognized at $P < 0.05$.

4.3 Results.

Under basal conditions and in the presence of 1.27 mM Ca^{2+} in the medium, mean arterial and venous P_{O_2} were 670.8 ± 5.2 and 363.7 ± 10.2 mmHg, respectively ($n = 47$) with a $\dot{V}_{O_2}$ of 7.1 ± 0.2 μmol.h^{-1}.g^{-1}, and perfusion pressure was 21.6 ± 0.5 mmHg ($n = 47$).

The transition from 1.27 mM to the lower [Ca^{2+}] caused a transient spike in perfusion pressure which returned to basal within 40 min in each perfusion (Table 4.1). The size of the pressure peak was less when medium containing 0.1 mM Ca^{2+} was added, but not significantly different to the peak observed with the other Ca^{2+} perturbations (one-way analysis of variance). Once perfusion pressure had returned to basal, there was no significant difference between basal perfusion pressure between Ca^{2+} perturbations (Table 4.1).

$[Ca^{2+}]^*$ (mM)	Perfusion Pressure (mmHg)		$\dot{V}O_2$ ($\mu mol.h^{-1}.g^{-1}$)		*n*
	Peak	Basal	Basal	Change	
1.27		21.6 ± 0.5	7.1 ± 0.2		47
0.10	35.0 ± 2.6	24.0 ± 1.7	6.4 ± 0.3	-1.3 ± 0.8	3
0.003	52.0 ± 4.3	25.5 ± 1.3	7.0 ± 0.4	-1.8 ± 0.7	4
Ca^{2+}-free	48.0 ± 2.7	20.6 ± 0.3	6.8 ± 0.2	-0.8 ± 0.2	15

Table 4.1 Effect of perfusion medium Ca^{2+} perturbations on basal hindlimb conditions.

Ca^{2+} with or without EGTA was added to the medium as described in the Methods. $\dot{V}O_2$, oxygen uptake, values obtained at steady state; *n*, no. hindlimb perfusions.

*, nominal extracellular $[Ca^{2+}]$.

4.3.1 NE-mediated effects.

Vasoconstriction stimulated by NE dose-dependently increased perfusion pressure but had a biphasic $\dot{V}O_2$ response in the rat hindlimb (Figure 4.2, described in detail in Chapter 2). Vasoconstriction stimulated by low doses of NE was associated with increases in $\dot{V}O_2$ (< 0.25 μM) and higher doses of NE began to inhibit the increase in $\dot{V}O_2$ towards basal (Figure 4.2). In some perfusions (Figures 4.3-4.5) propranolol was present in the perfusion buffer to inhibit β-AR-mediated vasodilatation (Chapter 2, Figure 2.10).

4.3.1.1 Perfusion medium Ca^{2+} perturbations.

Each reduction in extracellular [Ca^{2+}] progressively shifted the NE dose-response curve to the right and attenuated the maximum rise in perfusion pressure (Figure 4.2). Decreasing [Ca^{2+}] selectively attenuated the component of NE-mediated vasoconstriction associated with increases in $\dot{V}O_2$. When Ca^{2+} was eliminated from the perfusion medium, the NE-induced dose-dependent increases in perfusion pressure were immediately associated with decreases in $\dot{V}O_2$ (Figure 4.5). The maximal dose of NE (25 μM) increased perfusion pressure by 16.3 ± 1.8 mmHg and inhibited $\dot{V}O_2$ by 3.1 ± 0.7 $\mu mol.h^{-1}.g^{-1}$ ($n = 3$).

4.3.1.2 Effects of a_1-AR antagonists on the response to NE in the presence of extracellular Ca^{2+}.

Both 5-MU and CEC shifted the NE pressure response curve to the right and reduced the maximum pressure obtained (Figure 4.3). CEC (10 μM) shifted the NE $\dot{V}O_2$ response curve to the right without affecting the maximum $\dot{V}O_2$, yet the inhibitory effect of NE on $\dot{V}O_2$ was still observed. 5-MU (0.25 μM) shifted the NE $\dot{V}O_2$ response to the right, but only stimulation of $\dot{V}O_2$ was observed. The maximum $\dot{V}O_2$ was not altered, and increasing the NE dose did not lead to inhibition of $\dot{V}O_2$. Higher doses of CEC (100 μM) and 5-MU (1 μM) began to reduce the maximum $\dot{V}O_2$ (and pressure) response to NE (data not shown).

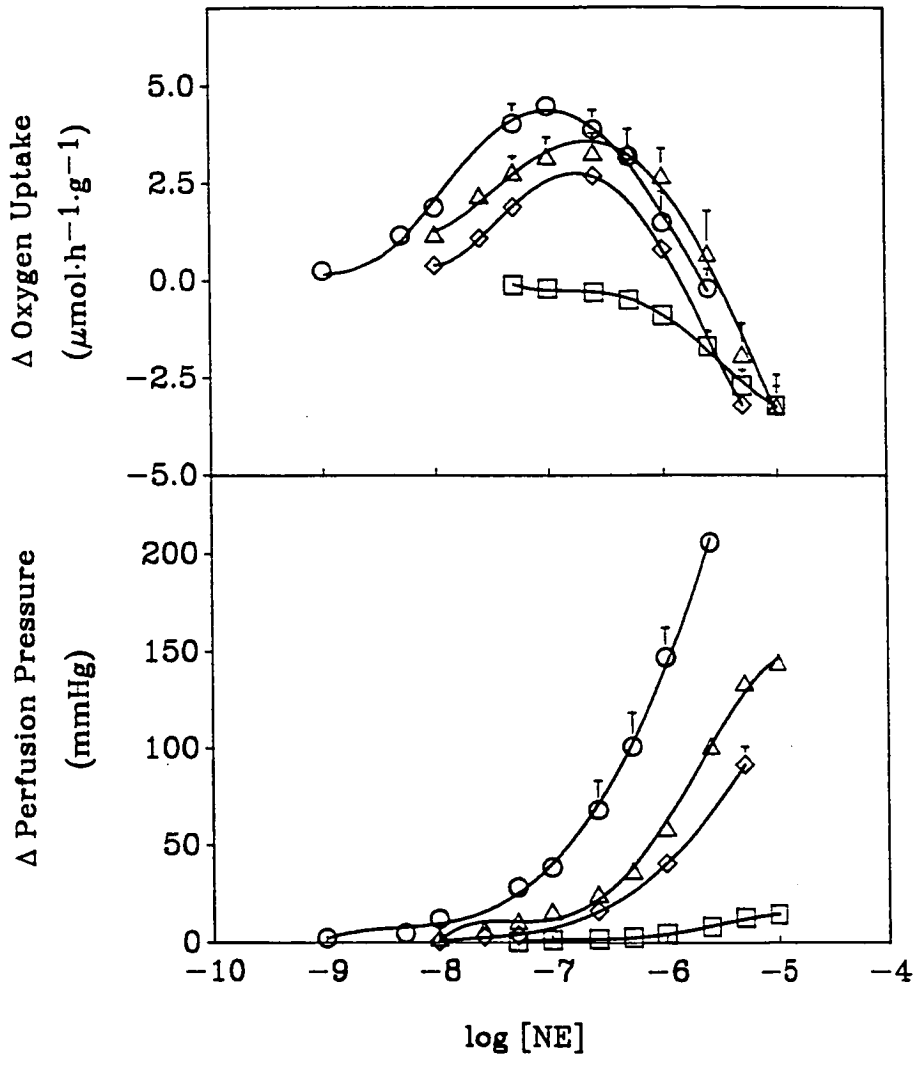

Figure 4.2 Effect of varying the extracellular $[Ca^{2+}]$ on the response to NE on hindlimb $\dot{V}O_2$ and perfusion pressure.

Hindlimbs from 180-200 g rats were perfused at 25°C and a constant flow rate of 4 $ml.min^{-1}$ with 1.27 mM Ca^{2+} (O), 0.1 mM Ca^{2+} (△), 1.27 mM Ca^{2+} and 1.27 mM EGTA (◇), or no Ca^{2+} plus 0.1 mM EGTA (□) added to the perfusion medium. Values are means ± SE of at least 3 hindlimbs for each Ca^{2+} perturbation. When not visible, error bars are within symbols. NE, norepinephrine; $\dot{V}O_2$, oxygen uptake.

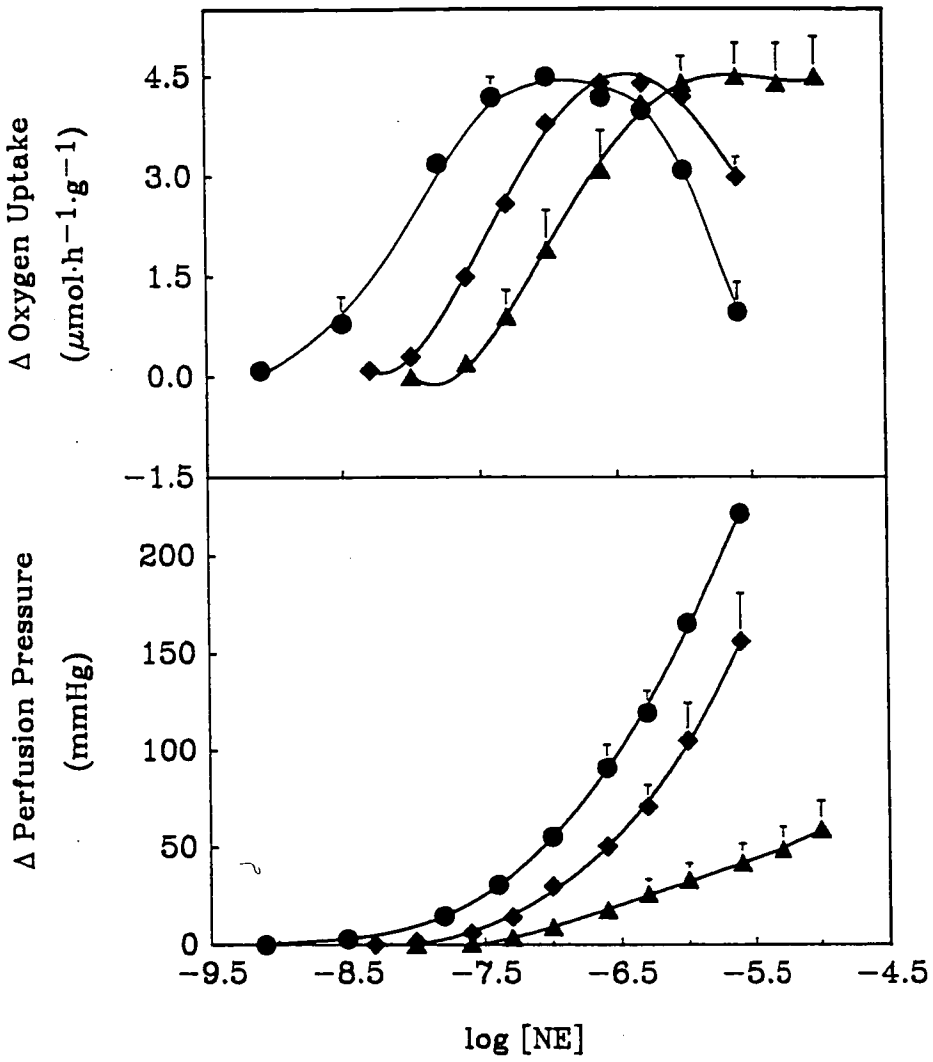

Figure 4.3 **Effect of α_1-AR antagonists on the dose-response curves for NE on hindlimb $\dot{V}O_2$ and perfusion pressure in the presence of extracellular Ca^{2+}.**

Hindlimbs were perfused as described in Figure 4.2 with medium containing 1.27 mM Ca^{2+} and 10 μM (±)-propranolol. NE was infused in the absence (●) and presence of 0.25 μM 5-MU (▲) or 10 μM CEC (◆). Values are means ± SE of at least 3 hindlimbs for each treatment. When not visible, error bars are within symbols. AR, adrenoceptor; $\dot{V}O_2$, oxygen uptake; NE, norepinephrine; CEC, chloroethylclonidine; 5-MU, 5-methyl urapidil.

Although both 5-MU and CEC reduced the magnitude of NE-mediated changes in pressure and $\dot{V}O_2$, the relationship between the change in $\dot{V}O_2$ and perfusion pressure was not affected, and interestingly fitted the same curve (Figure 4.5).

4.3.1.3 Effects of a_1-AR antagonists on the NE response in the absence of extracellular Ca^{2+}.

After equilibration in Ca^{2+}-free conditions, hindlimbs were treated with the same doses of antagonists in the same manner as when Ca^{2+} was present. Both CEC (10 μM) and 5-MU (0.25 μM) almost totally opposed the NE-mediated response (Figure 4.4).

4.3.2 5-HT-mediated effects.

Vasoconstriction stimulated by 5-HT dose-dependently directly inhibited $\dot{V}O_2$ in the rat hindlimb (Figure 4.6, and Chapter 2).

4.3.2.1 Perfusion medium Ca^{2+} perturbations.

When the perfusion medium [Ca^{2+}] was reduced, the $\dot{V}O_2$ and pressure response to 0.25 μM 5-HT was slightly augmented when 0.1 mM Ca^{2+} was added to the medium, but generally not affected (Table 4.2). In Ca^{2+}-free perfusions, however, 5-HT mediated rises in perfusion pressure were attenuated without affecting the decrease in $\dot{V}O_2$ (Figure 4.6).

4.3.3 Additivity of NE- and 5-HT-mediated effects.

In extracellular Ca^{2+}-free perfusions, both NE and 5-HT dose-response curves reached maximal values (Figures 4.4 & 4.6). When infused separately, 25 μM NE and 5 μM 5-HT induced similar maximal rises in perfusion pressure and inhibition of $\dot{V}O_2$. The maximal additive increases in perfusion pressure were 16.3 ± 4.0 mmHg and decreases in $\dot{V}O_2$ were 4.0 ± 0.6 $\mu mol.h^{-1}.g^{-1}$ (n = 4), and were independent of agonist infusion order (Figure 4.7). These preliminary results suggest the NE and 5-HT responses were only partially additive, and warrant further investigation.

$[Ca^{2+}]$* (mM)	$\Delta \dot{V}O_2$ ($\mu mol.h^{-1}.g^{-1}$)	Δ Perfusion Pressure (mmHg)	*n*
1.27	-1.5 ± 0.2	26.1 ± 3.2	8
0.10	-1.9 ± 0.3	37.8 ± 2.6#	3
0.003	-1.6 ± 1.2	26.9 ± 7.3	3
Ca^{2+}-free	-1.4 ± 0.2	7.8 ± 0.8#	6

Table 4.2 Effect of perfusion medium Ca^{2+} perturbations on the hindlimb response to 5-HT.

Rat hindlimbs were perfused at a constant flow rate of 4 $ml.min^{-1}$ with varying amounts of Ca^{2+} added to the perfusion medium as described in Table 4.1. Values for changes above basal mediated by 0.25 μM 5-HT are means ± SE. $\dot{V}O_2$, oxygen uptake; 5-HT, serotonin; *n*, no. of hindlimb perfusions; *, nominal extracellular $[Ca^{2+}]$; #, significantly different ($P < 0.05$) from response with 1.27 mM Ca^{2+}, unpaired Students *t* test.

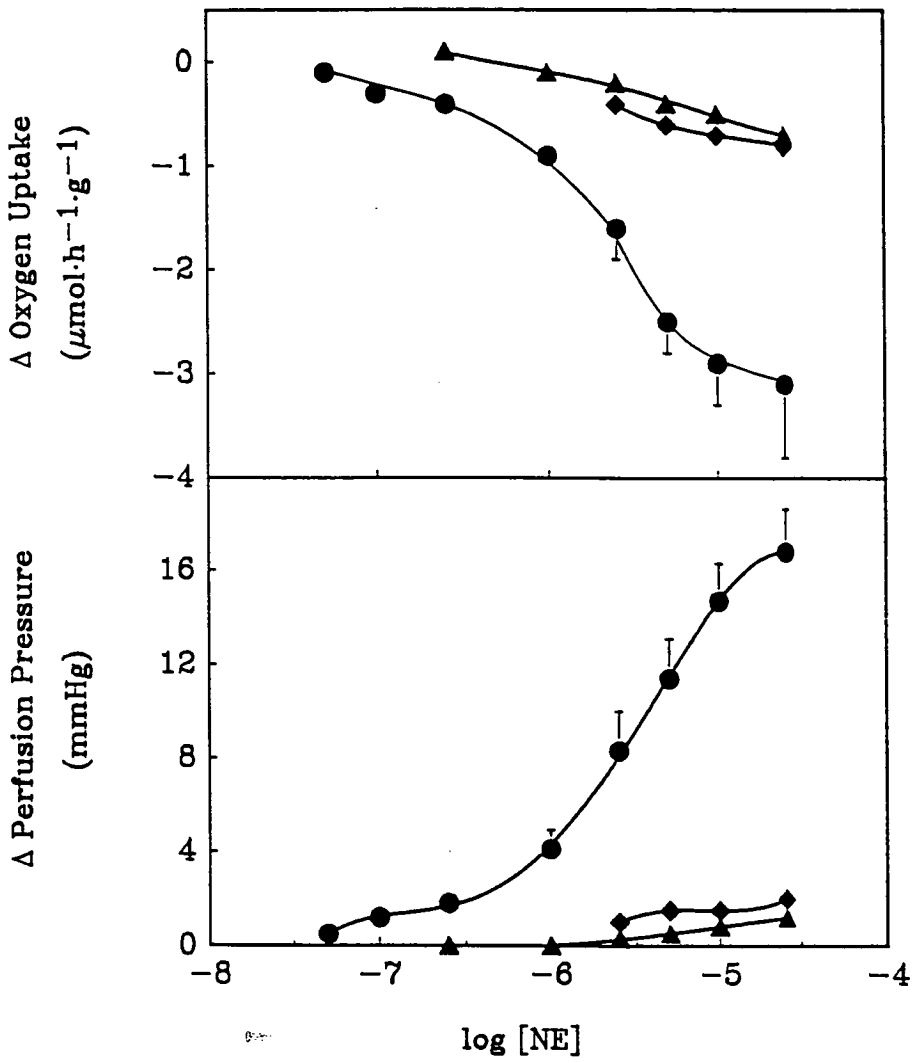

Figure 4.4 **Effect of α_1-AR antagonists on the dose-response curves for NE on hindlimb $\dot{V}O_2$ and perfusion pressure in the absence of extracellular Ca^{2+}.**

Hindlimbs were perfused as described in Figure 4.2 with Ca^{2+}-free medium containing 0.1 mM EGTA and 10 μM (±)-propranolol. NE was infused in the absence (●) and presence of 5-MU (▲, 0.25 μM) or CEC (◆, 10 μM). Values are means ± SE of at least 3 hindlimbs for each treatment. When not visible, error bars are within symbols. AR, adrenoceptor; $\dot{V}O_2$, oxygen uptake; NE, norepinephrine; CEC, chloroethylclonidine; 5-MU, 5-methyl urapidil.

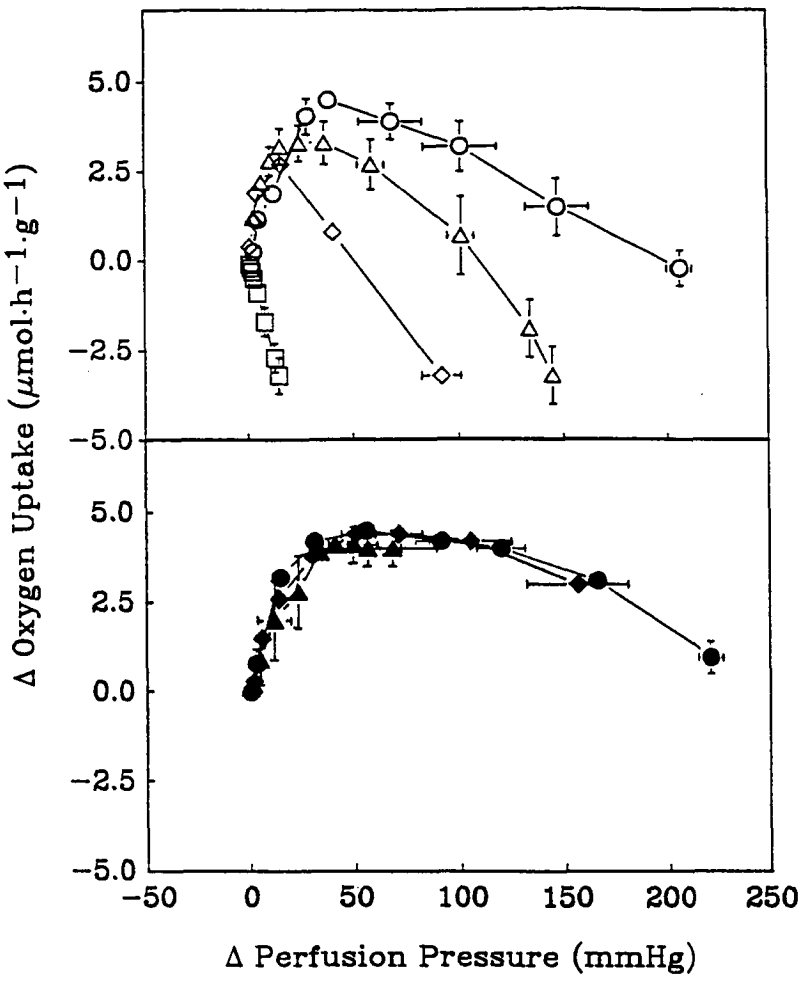

Figure 4.5 Dose-dependent effects of NE on changes in hindlimb $\dot{V}O_2$ *versus* changes in perfusion pressure.

Hindlimbs were perfused as described in Figure 4.2 in the absence (upper panel, data from Figure 4.2) or presence (lower panel, data from Figure 4.3) of 10 μM (±)-propranolol. The upper panel shows the NE dose-response curves when perfusion medium [Ca^{2+}] was varied by adding 1.27 mM Ca^{2+} (○), 0.1 mM Ca^{2+} (△), 1.27 mM Ca^{2+} and 1.27 mM EGTA (◇), or no Ca^{2+} and 0.1 mM EGTA (□) to the perfusion medium. The lower panel shows the NE dose-response curves in the absence (●) and presence of the selective α_1-AR antagonists 5-MU (▲, 0.25 μM) or CEC (◆, 10 μM). Values are means ± SE of at least 3 hindlimbs for each treatment. When not visible, error bars are within symbols. AR, adrenoceptor; $\dot{V}O_2$, oxygen uptake; NE, norepinephrine; CEC, chloroethylclonidine; 5-MU, 5-methyl urapidil.

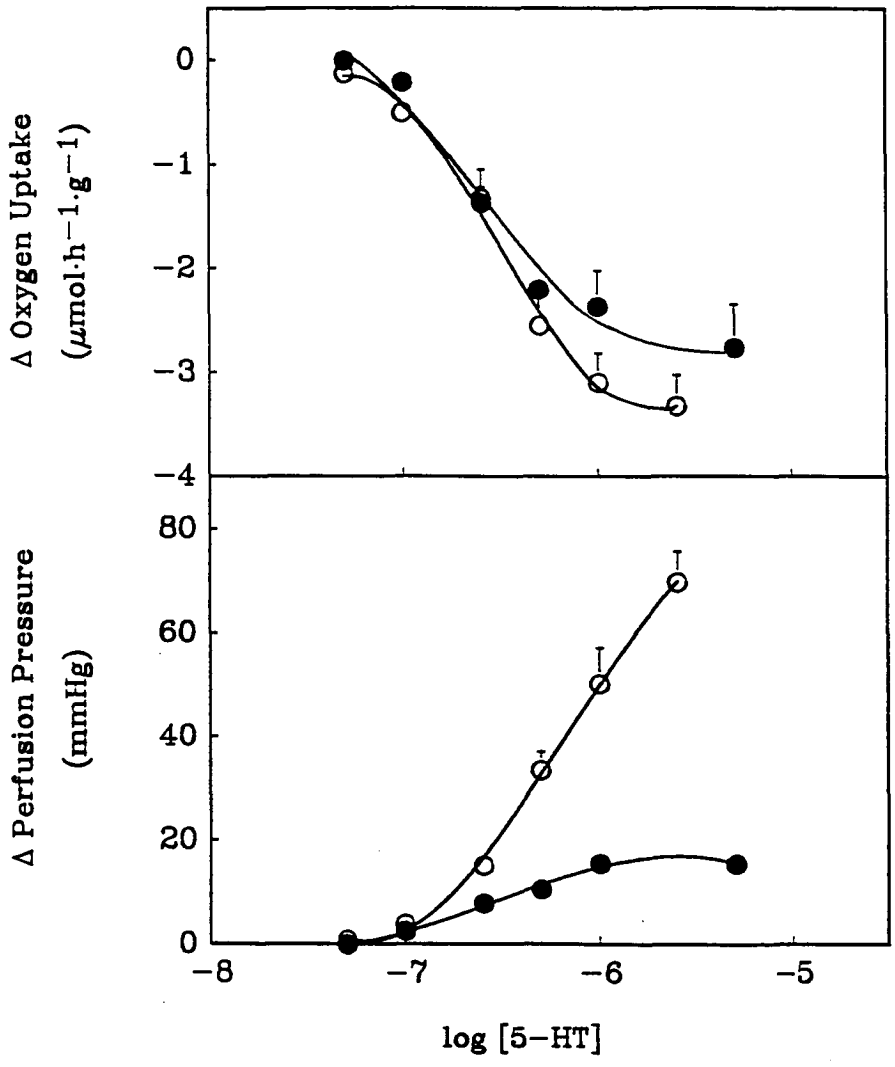

Figure 4.6 Effect of removing extracellular Ca^{2+} on the response to 5-HT on hindlimb $\dot{V}O_2$ and perfusion pressure.

Hindlimbs were perfused as described in Figure 4.2 with 1.27 mM Ca^{2+} (O) or no Ca^{2+} and 0.1 mM EGTA (●) added to the perfusion medium. Values are means ± SE of at least 3 hindlimbs for each [Ca^{2+}]. When not visible, error bars are within symbols. $\dot{V}O_2$, oxygen uptake; 5-HT, serotonin.

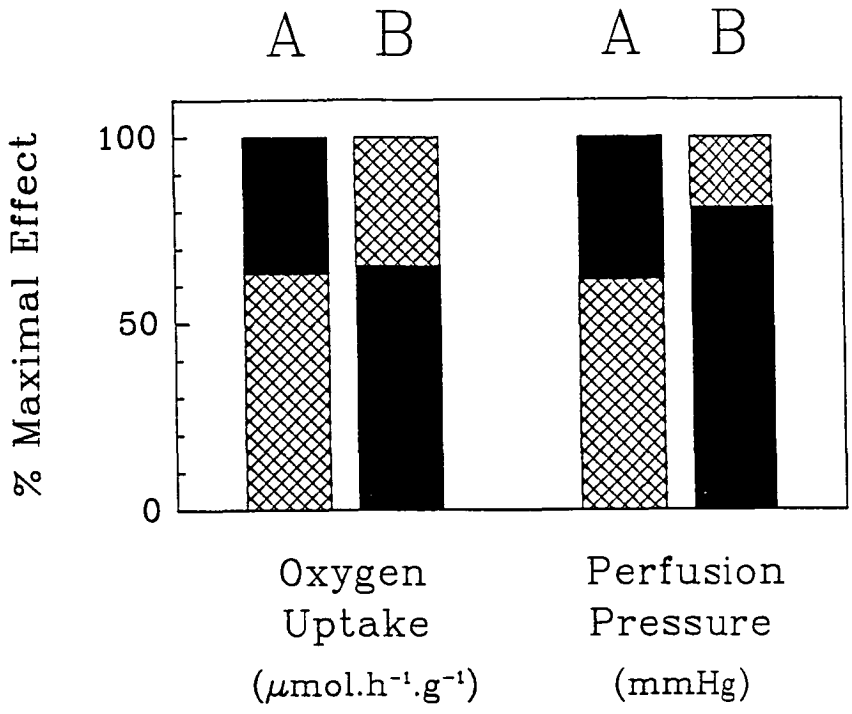

Figure 4.7 Additivity of maximal NE and 5-HT responses on hindlimb $\dot{V}O_2$ and perfusion pressure in the absence of extracellular Ca^{2+}.

Hindlimbs were perfused as described in Figure 4.2 with no Ca^{2+} and 0.1 mM EGTA added to the perfusion medium. Basal $\dot{V}O_2$ was 7.6 ± 0.7 μmol.h^{-1}.g^{-1} and perfusion pressure 22.3 ± 0.8 mmHg. When infused separately, maximal doses of NE (25 μM) and 5-HT (5 μM) stimulated similar rises in perfusion pressure and decreases in $\dot{V}O_2$; (Figures 4.4 and 4.6). When infused in the presence of each other, agonist infusion orders were either: (**A**) a maximal dose of NE (▩) followed by a maximal dose of 5-HT (■) (n = 2); or (**B**) a maximal dose of 5-HT followed by a maximal dose of NE (n =2). Values are presented as a proportion of the total combined response with two hindlimbs for each treatment sequence. The total responses on $\dot{V}O_2$ and perfusion pressure were -4.5 ± 1.3 and -3.4 ± 0.2 μmol.h^{-1}.g^{-1}, and 20.8 ± 7.2 and 11.8 ± 1.8 mmHg, respectively, for NE added before and after 5-HT. NE, norepinephrine; 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

4.4 Discussion.

These experiments provide further evidence for the ability of vascular smooth muscle to respond differently to low doses of NE and 5-HT. It was demonstrated that removal of Ca^{2+} from the perfusion medium resulted in the abolition of the vasoconstriction and associated increase in $\dot{V}O_2$ normally induced by NE at doses below 100 nM. 5-HT and doses of NE greater than 100 nM induced extracellular Ca^{2+}-independent vasoconstriction which was markedly reduced in comparison to normal Ca^{2+} perfusions. Both the NE and 5-HT-induced vasoconstriction in the absence of extracellular Ca^{2+} was associated with decreases in $\dot{V}O_2$.

It has previously been shown that the Ca^{2+}-channel antagonist nifedipine almost totally blocked the response to a low dose of NE in the constant-flow perfused rat hindlimb (Colquhoun *et al.*, 1990). In this study Ca^{2+} channel antagonists were not utilized due to the multiplicity of their effects, including blockade of α-ARs (reviewed in Smith, 1983). However, a disadvantage of removing perfusion medium Ca^{2+} was the possibility of internal Ca^{2+} store depletion. IP_3-signalled release of Ca^{2+} from the sarcoplasmic reticulum usually stimulates Ca^{2+}-influx and replenishment of internal Ca^{2+}-stores (Putney, 1987). Problems associated with extracellular Ca^{2+}-removal were demonstrated by Gouw *et al.* (1989) when the response to 5-HT in rat aorta was slightly attenuated by Ca^{2+}-entry blocking agents, but markedly reduced in Ca^{2+}-free medium. In Ca^{2+}-free conditions internal Ca^{2+}-stores were depleted and responses to agonists were time-dependent (Gouw *et al.*, 1989). This possibility of time dependency was not fully addressed in this study, although the responses to a maximal dose of 5-HT were maintained for a 60 min infusion period (data not shown), and dose-response curves for each agonist were usually completed within 60 min.

The 5-HT-mediated rises in perfusion pressure were markedly attenuated in Ca^{2+}-free medium, yet the decreases in $\dot{V}O_2$ were only slightly affected. The partial dissociation of these two events suggests the attenuated pressure response is unlikely to be explained by depletion of Ca^{2+}-stores. It appears that the vasoconstriction directly associated with the inhibition of $\dot{V}O_2$ was not affected, suggesting the mechanism for the inhibitory effect on $\dot{V}O_2$ was controlled by and associated with small changes in perfusion pressure. The dilatation mediated by orphan 5-HT-receptors (described in Chapter 2, Section 2.3.2) was not investigated in Ca^{2+}-free conditions, but it remains possible that both constriction and dilatation were

associated with the inhibition of $\dot{V}O_2$. There is growing evidence for at least two 5-HT receptors stimulating vasoconstriction in skeletal muscle (Alsip & Harris, 1991; Sumner *et al.*, 1992). Evidence suggests that these receptors may be distinguished on their Ca^{2+}-dependence for vasoconstriction. In dog saphenous vein, vasoconstriction mediated by stimulation with low doses of 5-HT, and stimulation of 5-HT_1-like receptors with the agonist sumatriptan was dependent on extracellular Ca^{2+}, whereas the response to higher doses of 5-HT was not (Sumner *et al.*, 1992). These workers suggested stimulation of 5-HT_1-like receptors was associated with both an influx of Ca^{2+}, predominantly via voltage-dependent channels, and an inhibition of adenylate cyclase (Sumner *et al.*, 1992). The higher dose 5-HT response would presumably be due to stimulation of 5-HT_{2A}-receptors coupled to IP_3-formation and Ca^{2+}-release from intracellular stores (Doyle *et al.*, 1986). In this study it appears that stimulation of 5-HT_{2A}, but not 5-HT_1-like receptors leads to vasoconstriction associated with inhibition of $\dot{V}O_2$. This possibility could be further investigated by infusion of sumatriptan into the hindlimb both in the presence and absence of extracellular Ca^{2+}.

The NE-stimulated biphasic $\dot{V}O_2$ response could also be separated by use of Ca^{2+}-free medium. Han *et al.* (1987), showed that stimulation of α_{1A}-AR caused an influx of Ca^{2+} into cells via G-proteins coupled to receptor-operated Ca^{2+} channels, whereas stimulation of α_{1B}-AR resulted in Ca^{2+} release from the sarcoplasmic reticulum via IP_3 formation. In this study, their classification scheme suggests that the observed NE-stimulated vasoconstriction associated with increases and decreases in $\dot{V}O_2$ were due to α_{1A}-AR- and α_{1B}-AR activation, respectively.

Agonist-mediated vasoconstriction that was blocked by receptor-operated Ca^{2+}-channel antagonists (*eg.* nifedipine) or may have been associated with IP_3-induced intracellular Ca^{2+} release is consistent with pharmacomechanical coupling (see Chapter 1, Section 1.2.1.2.1 for a description of the forms of excitation-contraction coupling). However in this study, IP_3 generation was not determined, thus allowing the possibility of electromechanical coupling with high doses of NE and/or 5-HT. This raises the possibility that the different metabolic (Chapter 3) and Ca^{2+}-dependencies of vasoconstriction are due to the compartmentalization of vascular smooth muscle metabolism. Glycolytic metabolism has been shown to be associated with plasma membrane ion motion (Paul *et al.*, 1979), and may provide energy for vasoconstriction stimulated via electromechanical coupling. Conversely, oxidative phosphorylation may supply energy during vasoconstriction stimulated via pharmacomechanical coupling.

There is accumulating evidence that classification of receptor subtypes solely according to extracellular Ca^{2+}-dependence may not be justified, as there appear to be more than two α_1-AR subtypes. Another approach to classify α_1-AR is use of selective α_1-AR antagonists. 5-MU and CEC have been shown to relatively selectively antagonize α_{1A}- and α_{1B}-AR, respectively, in rat tissues (Suzuki *et al.*, 1990; Han & Minneman, 1991). However, the results obtained in this study do not agree with the latter classification of α_1-AR. Both 5-MU and CEC had some affinity for the receptor stimulated by lower doses of NE, as in their presence the NE dose-response curves were shifted to the right. However the magnitude of the maximal increase in $\dot{V}O_2$ and the association between changes in $\dot{V}O_2$ and perfusion pressure remained unchanged (lower panel, Figure 4.5). A selective low-dose NE antagonist would be expected to alter this relationship in a manner similar to removal of extracellular Ca^{2+}, where small rises in perfusion pressure led to direct inhibition of $\dot{V}O_2$ (upper panel, Figure 4.5). Indeed, the results were opposite to expected in that 5-MU, but not CEC, blocked the inhibitory effect of NE on $\dot{V}O_2$[1]. In addition, both 5-MU and CEC inhibited the extracellular Ca^{2+}-independent vasoconstriction, despite earlier reports of 5-MU not interfering with NE-induced IP_3 formation (Suzuki *et al.*, 1990). These preliminary results suggest 5-MU was inhibiting a non α_{1A}-AR response according to the extracellular Ca^{2+}-dependency classification, and may represent a further subgroup of α_1-ARs.

Discrepancies in rat aorta α_1-AR sub-classification have been alluded to previously (Piascik *et al.*, 1991; Oriowo & Ruffolo, 1992). Oriowo and Ruffolo (1992) concluded that CEC and WB 4101 were not sufficiently reliable tools for α_1-AR subclassification, and proposed another subclass of α_1-AR in rat aorta. These authors also suggested α_1-AR could not be classified according to extracellular Ca^{2+}-dependence as proposed by Minneman (1988), and indeed have long proposed that a

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1. The conflicting results with CEC in the presence and absence of Ca^{2+} in the perfusion medium may represent a dose problem. Perhaps a higher dose of CEC (10 μM < [CEC] < 100 μM) was required to block the inhibitory effect of NE on $\dot{V}O_2$ when Ca^{2+} was present in the medium, as was expected if CEC selectively blocked extracellular Ca^{2+}-independent, α_{1B}-AR-stimulation. Ideally, Schild plots should be generated to determine the relative affinity of α_1-antagonists for the stimulatory and inhibitory NE-mediated responses on $\dot{V}O_2$. However the apparent problems of multiple receptor subtypes and the possible differences in receptor subtype population size would need to be addressed (methods reviewed by Kenakin, 1987).

single α_1-AR may activate two signal-transduction processes depending on the intrinsic efficacy of the agonist for the receptor (reviewed in Oriowo *et al.*, 1992). This possibility would warrant future investigation by careful use of the partial α_1-AR agonist dobutamine and the irreversible α_1-AR antagonist phenoxybenzamine as described by Oriowo *et al.* (1992).

However, this latter possibility is partially resolved and may be fully resolved in the future by molecular biology techniques. There is emerging evidence for intra- and inter-species heterogeneity of α_1-ARs (Lomasney *et al.*, 1991b; Schwinn *et al.*, 1990, 1991; reviewed by Lomasney *et al.*, 1991a and Roth *et al.*, 1991). Northern blot and *in situ* hybridization studies have shown that of the four cloned α_1-ARs (α_{1A}-, α_{1B}-, α_{1C}- and α_{1D}-AR) all except the α_{1C}-AR are expressed in rat tissues, with the α_{1A}-AR (and possibly α_{1D}-AR) being expressed in skeletal muscle (Lomasney *et al.*, 1990a; Perez *et al.*, 1991). The apparently low levels of skeletal muscle α_1-ARs may be explained by the observation that within skeletal muscle, α_1-ARs are expressed predominantly on arterial blood vessels and not skeletal myocytes (Rattigan *et al.*, 1986; Martin *et al.*, 1990). This would make detection of small, discreet populations of α_1-AR subtypes in skeletal muscle difficult, and thus pharamacological evidence should also be considered important.

Clones for the various α_1-ARs have been generated and expressed in cell lines to determine their respective signal-transduction pathways (reviewed by Lomasney *et al.*, 1991a). It is already well accepted that certain domains within sequences are characteristic of receptor signalling. In α_{1B}-AR the N-terminal portion of the third extracellular loop plays a major role in determining the selectivity of receptor-G protein coupling (Cotecchia *et al.*, 1992). All three α_1-AR subtypes are coupled to phospholipase C via a pertussis toxin-insensitive GTP-binding protein, leading to the release of Ca^{2+} from intracellular stores, although efficiency of coupling varies between subtypes (Schwinn *et al.*, 1991).

Despite the obvious differences in amino acid sequences between receptor subtypes, evidence is growing that a single receptor subtype can couple to multiple effector systems (Lomasney *et al.*, 1991a), supporting the earlier work by Ruffolo and co-workers (reviewed in Oriowo *et al.*, 1992). Insertion of the specific sequence conferring PI hydrolysis activity into the β_2-AR resulted in a chimeric AR which could activate both PI hydrolysis and adenylate cyclase (Cotecchia *et al.*, 1992). Thus direct comparisons can now be made between amino acid sequences which determine the selectivity of G protein coupling. However, expression would only be the first step and may not necessarily be of functional significance. If, for example, only one

α_1-AR was expressed in rat hindlimb vasculature but had sequences for both signal transduction pathways, then it must be assumed that heterogeneity in second-messenger-coupled processes, for example the presence of internal Ca^{2+}-stores, account for the varied $\dot{V}O_2$ responses.

It has been previously reported that small, resistance vessels were dependent on Ca^{2+} influx during vasoconstriction whereas larger vessels were not. In constant-flow perfused rat hindlimb the responsiveness of the microvasculature to NE indicated an increasing dependence on external Ca^{2+} as vessel size decreased towards capillaries (Sutter *et al.*, 1977). The NE-induced response in rabbit mesenteric resistance vessels was more sensitive to inhibitors of Ca^{2+} influx than the aorta (Cauvin & van Breeman, 1985; Cauvin *et al.*, 1985). In addition, only high doses of NE could stimulate extracellular Ca^{2+}-independent vasoconstriction in the aorta (Cauvin & Van Breeman, 1985; Cauvin *et al.*, 1985). In canine femoral artery the NE-induced contractile response was inhibited 70% by removing Ca^{2+} from the bathing solution (Eskinder *et al.*, 1989). In the same preparation, other workers (Koga *et al.*, 1989) reported that NE-induced vasoconstriction was prazosin-sensitive for all doses of NE in arteries, but only at high doses of NE in veins[2]. Hynes and Duling (1991) ascribed the general dependency of small vessels on extracellular Ca^{2+} to the rapid depletion of internal Ca^{2+}-stores in arterioles, at least partly due to the smaller proportion of sarcoplasmic reticulum per cell (Devine *et al.*, 1972; Ashida *et al.*, 1988), but also proposed smaller vessels had more active Ca^{2+} influx and efflux mechanisms, thus being more dependent on extracellular Ca^{2+}. In their cheek pouch preparation (in which they studied 60-90 μm perfused arterioles) the vasoconstrictor response to the α_1-AR agonist phenylephrine was abolished when the bathing solution [Ca^{2+}] was reduced to 10 μM (Hynes & Duling, 1991). These authors also reported a heterogeneity in response of the arterioles, as those responding to only high doses of phenylephrine were less sensitive to removal of medium Ca^{2+}. Thus it appears that a proportion of small vessels have greater than normal internal Ca^{2+}-stores.

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2. This raises the possibility that both high doses of NE and 5-HT also constricted veins. Many workers suggested veins were less extracellular Ca^{2+}-dependent for constriction than arteries (van Nueten & Vanhoutte, 1981). Sybertz *et al.* (1986) reported that rat hindlimb venous vasculature was more responsive to NE in Ca^{2+}-free perfusion than arterial vasculature. Using the present hindlimb perfusion method, it was not possible to assess venous constriction in this study.

The ability to categorize the $\dot{V}O_2$ effects according to extracellular Ca^{2+}-dependence provides further evidence that the low doses of NE were stimulating blood vessels metabolically distinct from those stimulated by high doses of NE and 5-HT. It was previously reported that the responses to these hormones could be separated when coupled mitochondrial oxidative phosphorylation was interrupted (Dora *et al.*, 1992, Chapter 3). Low doses of NE were totally dependent on oxidative metabolism whereas doses of NE greater than 50 nM, and all doses of 5-HT, were capable of anaerobic vasoconstriction. The results obtained here are consistent with the proposal that the thermogenic, low-dose (high-affinity) response to NE was due to constriction of extracellular Ca^{2+}-dependent resistance arterioles within the skeletal muscle microcirculation (Gray, 1971; Sutter *et al.*, 1977), whereas 5-HT was constricting larger blood vessels (Alsip & Harris, 1991; Sumner *et al.*, 1992) and causing a redistribution of flow such that thermogenic regions within the hindlimb received less flow (Dora *et al.*, 1991, 1992). Since high doses of NE caused an inhibition of $\dot{V}O_2$ in the absence of extracellular Ca^{2+}, it seems likely that the constriction sites for NE were also on larger blood vessels (Sutter *et al.*, 1977). The possibility that both NE and 5-HT stimulated vasoconstriction at the same anatomical location was partially addressed by examination of the additivity of effects in Ca^{2+}-free conditions. From the preliminary studies it appeared that the vasoconstriction induced by the two hormones were only partly additive, suggesting some homogeneity in stimulation mechanism and location.

Although plasma Ca^{2+} is never fully removed *in vivo*, extracellular Ca^{2+}-dependency of vasoconstriction has provided a useful method of dissociating the effects of NE on $\dot{V}O_2$. Whether NE was stimulating subgroups of α_1-ARs with different signal-transduction mechanisms or the same α_1-AR located on different blood vessels is a moot point. The ability of NE to cause the differential effects on $\dot{V}O_2$ has important implications *in vivo*. Access of O_2, nutrients and hormones to skeletal muscle may be controlled by the level of tone on different blood vessels. Alterations in tone, be it from circulating or neuronal sources, and sensitivity to vasoactive agents in disease states such as hypertension and atherosclerosis may compromise tissue need and be responsible for conditions associated with these diseases.

In summary, the receptors stimulated by NE and 5-HT may be sub-classified according to their extracellular Ca^{2+}-dependence and the use of selective receptor antagonists. Table 4.3 outlines the evidence supporting each receptor sub-classification.

Agonist ± Antagonist	Effect on: Pressure	Effect on: $\dot{V}O_2$	Dependence on: Aerobic Metabolism‡	Dependence on: $[Ca^{2+}]_o$	Selective Antagonists	**Proposed Receptor**
NE+Prop						
low-dose	↑	↑	yes	yes	?	$\alpha_{1A(like)}$
high-dose	↑	↓	no	no	Prazosin† 5-MU CEC	$\alpha_{1B(like)}$
NE+Praz						
low-dose	nil	nil				
high-dose	↓	↓	*	slight *	Propranolol	β
5-HT						
low/high	↑	↓	no	no	LY 53,857†	5-HT_{2A}
high-dose	↑	*	no	yes	LY 53,857	5-HT_1-like?
5-HT+LY						
low/high	↓	↓	*	*	*	orphan 5-HT

Table 4.3 Summary of evidence for sub-classification of AR- and 5-HT-receptor stimulation in constant-flow perfused rat hindlimb.

Results indicative of receptor sub-division from this Chapter and Chapters 2 (†) and 3(‡) are summarized. AR, adrenoceptor; $\dot{V}O_2$, oxygen uptake; $[Ca^{2+}]_o$, extracellular Ca^{2+}; NE, norepinephrine; low-dose NE, < 0.1 μM NE; 5-HT, serotonin; low-dose 5-HT, < 0.25 μM 5-HT; CEC, chloroethylclonidine; 5-MU, 5-methyl urapidil; LY, LY 53,857; *, not addressed in this study, warrants future investigation.

CHAPTER 5

Effect of vasoconstrictors on insulin-mediated glucose uptake.

5.1 Introduction.

The effects of vasoconstrictors on hindlimb metabolism has been limited to determination of oxygen uptake ($\dot{V}O_2$) and lactate release (Chapter 3). However, the metabolism of glucose is more frequently associated with skeletal muscle. Insulin (James *et al.*, 1988) and exercise (Douen *et al.*, 1990) have both been shown to translocate the unique GLUT 4 glucose transporters to skeletal myocyte cell membranes from distinct internal stores (Douen *et al.*, 1990; Marette *et al.*, 1992) by an as yet not fully determined mechanism.

The rat hindlimb and representative incubated muscles have both been used to determine the effects of hormones on skeletal muscle glucose transport, disposal and net uptake, usually employing various radiolabelled analogues of glucose (James *et al.*, 1985; Wallberg-Henriksson *et al.*, 1988). In the constant-flow perfused hindlimb, the effects of epinephrine (Chiasson *et al.*, 1981; Richter *et al.*, 1982a), insulin (Ruderman *et al.*, 1971, 1977; Chiasson *et al.*, 1981; Schadewaldt *et al.*, 1985) and the combination of epinephrine and insulin (Chiasson *et al.*, 1981; Richter *et al.*, 1982a) on glucose uptake have been determined.

The above studies clearly indicated an epinephrine-mediated increase in hindlimb glucose uptake, although Chiasson *et al.* (1981) ascribed the uptake to β-adrenoceptor (AR) stimulation, whereas Richter *et al.*, (1982a) proposed an α-AR stimulation. Upon further investigation, Chiasson *et al.* (1981) found a lack β-AR stimulation of glucose uptake into striated muscle, which was consistent with the results from isolated, incubated soleus muscles (Sloan *et al.*, 1978). Thus Chiasson *et al.* (1981) proposed that in the hindlimb epinephrine was stimulating glucose uptake into adipose tissue. However, both β-AR mediated rises in cAMP (van Putten & Krans, 1985) and stimulation of protein kinase C (Saltis *et al.*, 1991) have been shown to increase adipocyte glucose uptake, thus leaving the issue as to which AR subtype was responsible for increasing hindlimb glucose uptake in some doubt.

Insulin has been shown by many groups to increase glucose uptake into hindlimb tissue (Ruderman *et al.*, 1971; Chiasson *et al.*, 1981; James *et al.*, 1985;

Kubo & Foley, 1986; Klip *et al.*, 1987). Insulin also increases glycogen synthesis by directly stimulating glycogen synthase activity.

The inhibitory effect of epinephrine on insulin-stimulated glucose uptake is generally accepted to be due to β-AR stimulation. Chiasson *et al.*, 1981 showed that epinephrine, via β- but not α_1-adrenoceptor (AR) stimulation, caused an attenuation of insulin-stimulated glucose uptake. β-AR stimulation is believed to oppose the actions of insulin by increasing the rate of glycogenolysis and thus causing an increase in tissue glucose 6-phosphate concentration, which in turn inhibits the rate of glucose phosphorylation without affecting the rate of glucose transport (Challiss *et al.*, 1986). Glucose uptake is decreased due to an increase in glucose transport out of cells (Chiasson *et al.*, 1981).

Both β-AR stimulation (Dimitriadis *et al.*, 1991; Hettiarachchi *et al.*, 1992) and insulin (Dimitriadis *et al.*, 1991) have been shown to increase lactate release from skeletal muscle preparations by increasing glycolytic activity, β-AR stimulation increasing glycogenolysis (Dimitriadis *et al.*, 1991) predominantly in fast-twitch white fibres (Richter *et al.*, 1982a) and insulin increasing cytosolic glucose. The combination of β-AR and insulin stimulation was not additive on lactate release (Challiss *et al.*, 1986; Dimitriadis *et al.*, 1991) presumably due to build up of glucose 6-phosphate. α-AR stimulation has been shown not to increase incubated skeletal muscle lactate release (Hettiarachchi *et al.*, 1992), although smooth muscle increases lactate production during constriction (Paul, 1980; Lundholm *et al.*, 1983; Hettiarachchi *et al.*, 1992).

Recent interest in the link between blood flow and skeletal muscle insulin-mediated glucose uptake has shown that in insulin-resistant patients, insulin-mediated increases in blood flow to skeletal muscle appears impaired (reviewed by Baron *et al.*, 1993). As insulin-mediated vasodilatation does not appear to involve a specific receptor (Wambach & Liu, 1992) it may be possible that the delivery of insulin (and glucose) to the skeletal muscle microcirculation may be reduced (reviewed by Julius *et al.*, 1992). Since both norepinephrine (NE) and serotonin (5-HT) have been shown to differentially affect metabolism of, and flow through, the hindlimb (Chapter 2, Dora *et al.*, 1991), we have examined the effects of 5-HT and NE on insulin-mediated glucose uptake in the constant-flow perfused rat hindlimb. A comparison was also conducted with isolated incubated muscles where nutrients were supplied by diffusion from the outside rather than through the vascular system.

5.2 Materials and methods.

5.2.1 Perfused hindlimbs.

This series of experiments could not be performed at 25°C due to difficulties in accurately measuring small basal and insulin-mediated glucose uptake at this temperature (Table 5.2). The increased rates of metabolism at higher temperatures necessitated the use of higher flow rates and 4% BSA. While using a non-recirculating system, it was decided that experiments would be performed at either 32°C with large rats or 37°C with small rats, thereby reducing cost, enabling accurate measurement of glucose-uptake and not compromising tissue oxygen delivery.

Surgery was performed on either 70-80 g or 240-270 g rats as outlined in Section 2.2.1.2.1, except both hindlimbs were perfused and flow rates increased (described in more detail in relevant sections). Experiments were performed at either 25, 32 or 37°C with 4% bovine serum albumin added to the perfusion medium. Additional details are given in Section 2.2.1.

$\dot{V}O_2$ was calculated as described in Section 2.2.1.6 using the appropriate Bunsen coefficient (Christoforides *et al.*, 1969). The effluent was periodically sampled (at times indicated) for measurement of glucose and lactate levels, or otherwise discarded. The hindlimbs were allowed to equilibrate for 30-35 min before venous sampling or hormone additions were commenced. Effluent lactate levels were determined according to the method of Gutmann and Wahlefeld (1974) as described in Section 2.2.2. Perfusate glucose concentration was determined using the GOD-Perid method (Boehringer Mannheim, Germany). Glucose uptake was calculated from the arterio-venous differences in perfusate glucose concentration multiplied by the perfusate flow rate divided by the weight of muscle.

5.2.1.1 Experiments involving 5-HT.

The temperature of the perfusion cabinet, heat exchanger, jacketed oxygen electrode and arterial inflow line were adjusted to 32°C and monitored throughout experiments. For each experiment, both hindlimbs from 240-270 g rats were perfused at a constant flow rate (15 $ml.min^{-1}$) with Krebs-Henseleit bicarbonate buffer containing 2.5 mM $CaCl_2$, 8.3 mM glucose and 4% bovine serum albumin (BSA). In these larger rats, perfused muscle mass was estimated at one-sixth of the rat's body

mass, consistent with Ruderman *et al.* (1971).

The dose of insulin was also chosen to give a maximal effect on glucose uptake (Chiasson *et al.*, 1981). Stock solutions of insulin (3 μM) in perfusion medium (with BSA), 5-HT (2.1 mM) in 0.1% ascorbic acid/0.9% NaCl or their vehicles were infused at the times indicated at a rate of 1 in 200 of the flow rate into the perfusion line prior to a combined small bubble trap and mixing chamber that was continuously mixed by a magnetic stirrer.

5.2.1.2 Experiments involving NE.

The temperature of the perfusion cabinet, heat exchanger, jacketed oxygen electrode and arterial inflow line were adjusted to 37°C and monitored throughout experiments. For each experiment both hindlimbs from 70-80 g rats were perfused at a constant flow (8 $ml.min^{-1}$) with Krebs-Henseleit bicarbonate buffer containing 2.5 mM $CaCl_2$, 8.3 mM glucose and 4% bovine serum albumin (BSA). Preliminary results showed that the relationship for hindlimb mass as a proportion of total body mass (Ruderman *et al.*, 1971) did not hold for small rats. Thus the mass of perfused muscle in the small rats was determined by injecting filtered Evans Blue dye at the end of control perfusions and weighing the dissected stained muscle to give the regression equation ($n = 6$):

perfused muscle mass (g) = 0.141 x body mass (g) - 2.53

Stock solutions of insulin (3 μM) in perfusion buffer, NE (0.2 or 2 mM) in 0.1% ascorbic acid/0.9% NaCl, (±)-propranolol (2 mM) in 0.9% NaCl or prazosin (0.5 mM) in 10% dimethyl sulfoxide $\{(CH_3)_2SO\}$ were infused at the times indicated at a rate of 1 in 200 of the flow rate into the perfusion line prior to a combined small bubble trap and mixing chamber that was continuously mixed by a magnetic stirrer.

5.2.2 Incubated muscles.

Soleus (20-30 mg) and extensor digitorum longus (20-30 mg) muscles were dissected from 60-80 g rats and attached (2 per experiment) to stainless steel springs to maintain their approximate *in vivo* length. The muscles were incubated in flasks containing 5 ml of Krebs-Ringer bicarbonate buffer of the same composition as used in the perfusion experiments. The flasks were maintained at 32°C and continuously

gassed with 95% O_2-5% CO_2. After 40 min the muscles were transferred to new flasks containing a fresh 5 ml of buffer and either the hormones at concentrations used in the perfusion experiments or their vehicles as indicated. After 20 min 2-deoxy-D-[1-^{3}H]glucose/[U-^{14}C]sucrose was added and the incubations continued for a total of 90 min. At the end of the incubation the muscles were removed, blotted dry, weighed and freeze-dried overnight. After reweighing to determine the dry weight the muscles were homogenized in 2 ml of water. The homogenate was centrifuged (8000 g for 15 min) and the supernatant counted for radioactivity. Samples of incubation medium were also counted for radioactivity. The R'g (μmol.h^{-1}.g^{-1}) was calculated from the following expression:-

$$\frac{2 \times [^3\text{H d.p.m. in muscle} - (^{14}\text{C d.p.m. in muscle} \times {}^3\text{H d.p.m.}/{}^{14}\text{C d.p.m. ratio in buffer})]}{\text{dry wt muscle (g)} \times (\mu\text{moles per ml glucose in buffer} / {}^3\text{H d.p.m. per ml in buffer})}$$

5.2.3 Statistical analysis.

The significance of difference between treatments of perfusions and incubations were tested either by one way analysis of variance and least significant difference analysis (Snedecor & Cochran, 1980) using the SAS statistical package (SAS Institute Inc., Cary, N.C.), or the unpaired, 2-tailed Student's *t* test. Values of $P < 0.05$ were taken as significant.

The significance of difference within treatments were tested using the paired, 2-tailed Student's *t* test.

5.3 Results.

Basal perfusion conditions for the various rat sizes, temperatures and flow rates are given in Table 5.1. Raising perfusion temperature markedly increased basal $\dot{V}O_2$ and lactate release. Rates of metabolism increased with decreasing rat size and increased with flow rate in accordance with previous findings at 25°C (Ye *et al.*, 1990b, Hettiarachchi *et al.*, 1992); and the results of others (Table 5.1).

Size (g)	Temp (°C)	Flow Rate (ml.min^{-1})	$\dot{V}O_2$ (μmol.h^{-1}.g^{-1})	Lactate Release (μmol.h^{-1}.g^{-1})	Perfusion Pressure (mmHg)	*n*
70-80	25	1.5*	12.9 ± 0.6	N.D.	22.3 ± 0.8	6
	25	4.0**	15.5 ± 0.6	10.0[#]	31.0 ± 1.5	2
	37	4.0**	23.7 ± 0.5	31.6 ± 1.2	28.6 ± 0.4	44
110-120	25[A]	1.2*	9.1 ± 0.7	8.4 ± 1.3	19.0 ± 0.7	5
180-200	25[B]	4.0*	6.6 ± 0.1	7.4 ± 0.5[†]	22.5 ± 0.4	65
	25[C]	4.0**	6.9 ± 0.2	4.1 ± 0.4[‡]	22.6 ± 0.6	27
	25[D]	16.0*	8.0 ± 0.5	6.6 ± 1.3	58.8 ± 1.2	20
	37[E]	5.0**	18.0 ± 2.4	4.8 ± 0.6	N.D.	8
	37[F]	16.0*	42.0 ± 15.0	9.0 ± 3.0	63.3 ± 1.3	8
240-270	32	7.5*	10.0 ± 0.2	11.6 ± 0.9	24.6 ± 0.4	20

Table 5.1 Relationship between rat size, perfusion temperature and flow rate on basal hindlimb perfusion conditions.

Perfusion medium contained 4% bovine serum albumin (BSA) and 2.5 mM Ca^{2+} in all experiments except those performed at 25°C which contained 2% BSA and 1.27 mM Ca^{2+}. Values are means ± SE. $\dot{V}O_2$, oxygen uptake; *n*, no. of hindlimb perfusions; N.D., not determined; A, from Hettiarachchi *et al.*, 1992; B, from Chapter 3; C, from Chapter 4; D, from Chapter 8; E, from Ruderman *et al.* (1971), medium contained erythrocytes; F, from Chapter 8, medium contained erythrocytes; flow rate as ml.min^{-1}.hindlimb^{-1}; *, one hindlimb perfused; **, two hindlimbs perfused; [#] $n = 1$; [†], $n = 5$; [‡], $n = 23$.

5.3.1 *Experiments involving 5-HT at 32°C.*

Consistent with our previous studies using hindlimbs perfused at 25°C (Chapter 2, Dora *et al.*, 1991), 5-HT increased perfusion pressure and inhibited $\dot{V}O_2$ in a dose-dependent manner at 32°C (Figure 5.1). The effects on $\dot{V}O_2$ and pressure were totally reversible upon removal of 5-HT. A maximal dose of 5-HT (10 μM) was used in this study. This series of experiments was performed prior to the development of surgical technique required for the 70-80 g rats used in experiments involving NE. Subsequent perfusions have shown that the response to 0.25 μM 5-HT at 25°C was also apparent in 80 g rats (decrease in basal $\dot{V}O_2$ of 7.0 $\mu mol.h^{-1}.g^{-1}$ associated with an increase in perfusion pressure of 35 mmHg, $n = 1$).

Figure 5.4 summarizes the average values obtained 50 min after commencement of treatment (termed "steady-state") in this study. In control perfusions, $\dot{V}O_2$ and perfusion pressure remained constant for the 90 min experimental period (Figure 5.2, left panels). The hindlimb perfusion pressure before additions was 24.6 ± 0.4 mmHg ($n = 20$) at the flow rate of 15 $ml.min^{-1}$. Under no conditions did venous PO_2 fall below 250 mmHg (arterial PO_2 was constant at 680 ± 3 mmHg, $n = 20$). However neither hindlimb lactate release nor glucose uptake were constant during control perfusions. Lactate release gradually increased from approx. 10 to 16 $\mu mol.h^{-1}.g^{-1}$ over the initial 20 min, then remained constant at approx. 14.5 $\mu mol.h^{-1}.g^{-1}$ for the remaining 35 min (Figure 5.3, upper left panel). Glucose uptake gradually increased from approx. 5 to 10 $\mu mol.h^{-1}.g^{-1}$ over the initial 10 minutes and then declined to settle at approx. 8 $\mu mol.h^{-1}.g^{-1}$ by 90 min (Figure 5.3, lower left panel).

5.3.1.1 *Effects of insulin in perfused hindlimbs.*

Insulin (15 nM) had no effect on perfusion pressure even though it caused a small increase in $\dot{V}O_2$ (8%) (Figure 5.2 left panels). Insulin markedly stimulated lactate release and glucose uptake to steady-state values 1.8-fold and 2.9-fold, respectively, above corresponding controls (Figure 5.3 left panel).

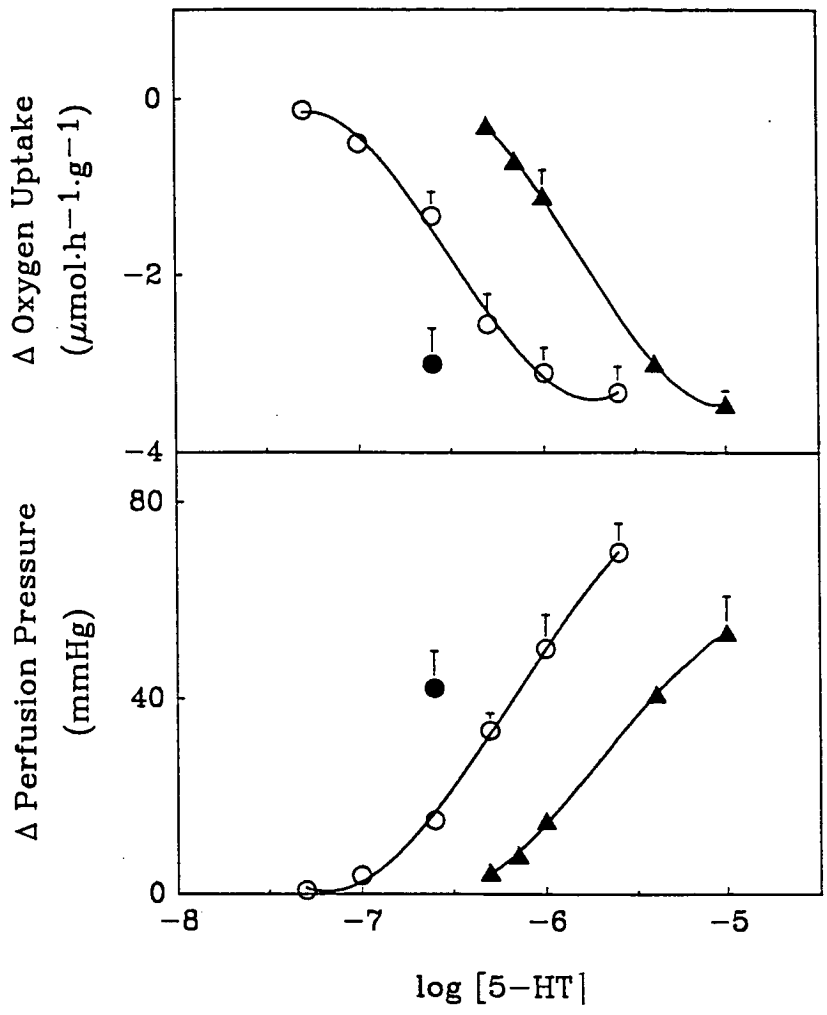

Figure 5.1 **Effects of perfusion temperature, flow rate and rat size on the dose-dependent $\dot{V}O_2$ and perfusion pressure effects of 5-HT in hindlimbs.**

Basal perfusion conditions are given in Table 5.1. Each dose of 5-HT was infused constantly for 10 min into either one hindlimb of 180-200 g rats perfused at 4 ml.min^{-1} and 25°C (○, n = 5, from Chapter 2) or 16 ml.min^{-1} and 25°C (●, n =3, from Chapter 6); or two hindlimbs of 250 g rats perfused at 15 ml.min^{-1} and 32°C (▲, n = 3). Values are means ± SE. 5-HT, serotonin; n, no. of hindlimb perfusions.

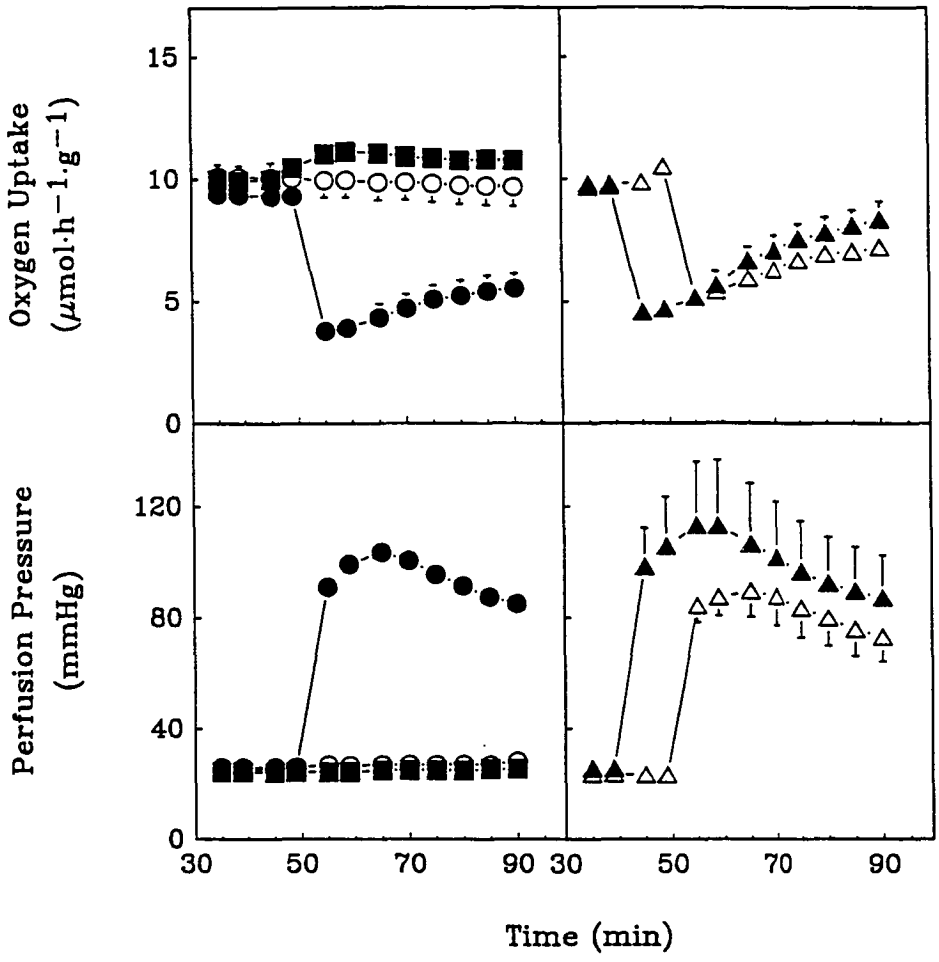

Figure 5.2 Effects of insulin, and 5-HT in the absence and presence of insulin, on hindlimb $\dot{V}O_2$ and perfusion pressure.

Both hindlimbs from 250 g rats were perfused at a constant flow rate of 15 $ml.min^{-1}$ at 32°C in a non-recirculating mode. The following constant infusions were commenced at the times stated below to give the final concentrations shown and these were maintained until the end of the perfusion: 50 min, vehicle (O); 50 min, 10 μM 5-HT (●); 40 min, 15 nM insulin (■); 40 min, 15 nM insulin and 50 min, 10 μM 5-HT (△); 40 min, 10 μM 5-HT and 50 min, 15 nM insulin (▲). Values shown are means ± SE for 4 perfusions. When not visible, the error bar is within the symbol. 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

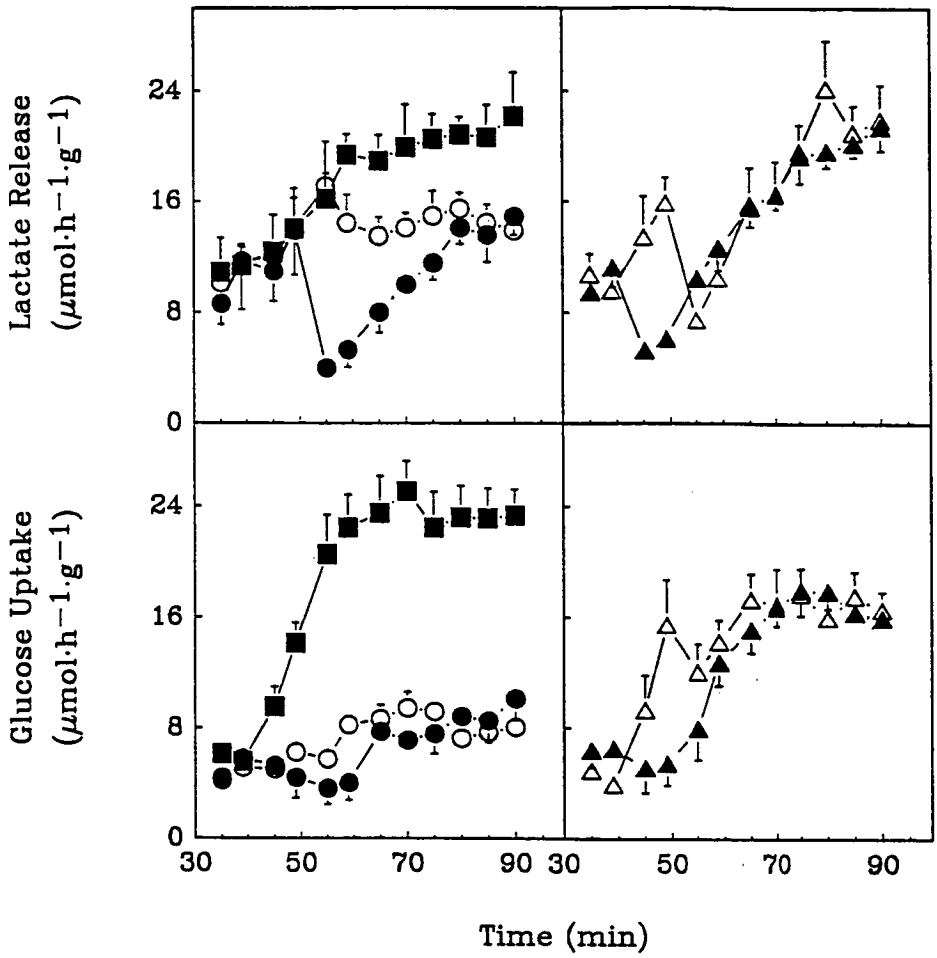

Figure 5.3 Effects of insulin, and 5-HT in the absence and presence of insulin, on hindlimb lactate release and glucose uptake.

Hindlimbs were perfused as described in Figure 5.2. The following constant infusions were commenced at the times stated below to give the final concentrations shown and these were maintained until the end of the perfusion: 50 min, vehicle (○); 50 min, 10 μM 5-HT (●); 40 min, 15 nM insulin (■); 40 min, 15 nM insulin and 50 min, 10 μM 5-HT (△); 40 min, 10 μM 5-HT and 50 min, 15 nM insulin (▲). The arterial perfusate concentration of glucose was 8.3 mM and glucose uptake was calculated from the arterio-venous difference from venous samples taken at the times shown. Values shown are means ± SE for 4 perfusions. When not visible, the error bar is within the symbol. 5-HT, serotonin.

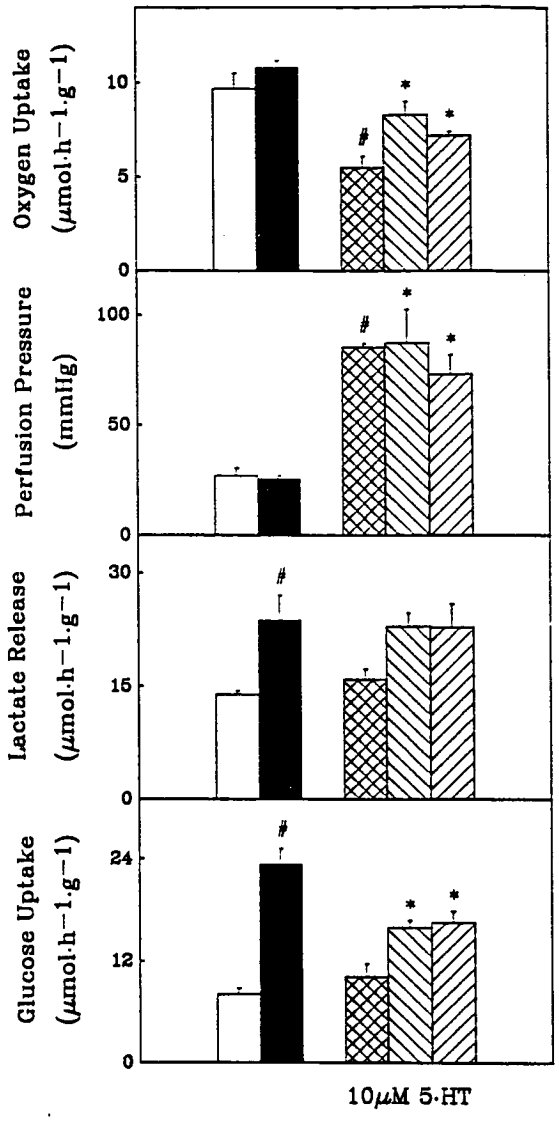

Figure 5.4 Average steady-state effects of insulin, and 5-HT in the presence and absence of insulin, on hindlimb $\dot{V}O_2$, perfusion pressure, lactate release and glucose uptake.

Data for the values obtained at the end of the perfusion period (50 min after commencement of treatment at t = 90 min) from Figures 5.2 and Figure 5.3 were averaged for control (□); 15 nM insulin (■); 5-HT (▩); 5-HT + 15 nM insulin (▧); 15 nM insulin + 5-HT (▨). Values are means ± SE of four perfusions for each treatment. Significance of differences ($P < 0.05$) were assessed using one-way ANOVA: #, control different from insulin or 5-HT; *, insulin different from 5-HT + insulin or insulin + 5-HT; 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

5.3.1.2 Effects of 5-HT in perfused hindlimbs.

The present effects mediated by 5-HT were sustained over the period of constant infusion, yet both pressure and $\dot{V}O_2$ did commence a gradual partial return towards basal levels. At 59 min perfusion pressure was 99.4 ± 3.1 mmHg (n = 4) and $\dot{V}O_2$ was 3.9 ± 0.5 $\mu mol.h^{-1}.g^{-1}$ (n = 4). By 90 min perfusion pressure had significantly ($P < 0.05$, paired Student's t test) decreased to 85.1 ± 1.8 mmHg (n = 4) and $\dot{V}O_2$ significantly ($P < 0.001$, paired Student's t test) increased to 5.5 ± 0.6 $\mu mol.h^{-1}.g^{-1}$ (n = 4) (Figure 5.2, left panels).

After 5 min of infusion, 5-HT initially markedly and significantly ($P < 0.01$, unpaired Student's t test) attenuated lactate release to levels only half that found under basal conditions. Over the remainder of the perfusion period, lactate release returned to control levels (Figure 5.3, upper left panel). Despite an initial trend of decreased glucose uptake, 5-HT did not significantly alter hindlimb glucose uptake compared to the vehicle perfusion (Figure 5.3, lower left panel).

5.3.1.3 Effects of insulin plus 5-HT in perfused hindlimbs.

Insulin did not alter the 5-HT-mediated vasoconstriction or inhibition of $\dot{V}O_2$, whether added before or after 5-HT. However it was apparent that insulin, when added after 5-HT, tended to augment the pressor effect but this was not a statistically significant effect due to an increase in variability (Figure 5.2).

When 5-HT was added following insulin, the stimulatory effect of the latter on lactate release and glucose uptake was reduced, and this was most apparent soon after 5-HT addition. Whether added before or after insulin, lactate release gradually returned to control levels (Figure 5.3, upper panels). If added before insulin, 5-HT decreased the insulin-mediated glucose uptake at all time points (Figure 5.3, lower panels). Infusion of 5-HT before or after insulin significantly inhibited steady-state insulin-mediated glucose uptake (Figure 5.4). Thus for perfusions where insulin was added first, 5-HT resulted in a 28.9% reduction in the glucose uptake stimulation due to insulin and for perfusions where 5-HT was added first, the stimulation was reduced by 31.6%.

5.3.1.4 *Effects of insulin, 5-HT, and insulin plus 5-HT in incubated muscles.*

Glucose uptake by isolated incubated soleus and extensor digitorum longus muscles is shown in Figure 5.5. Conditions of incubation were chosen to be identical to those of perfusion. However smaller rats were used to obtain muscles that weighed less than 30 mg to allow suitable oxygenation of the muscle during incubation. The medium contained 4% albumin and 8.3 mM glucose and pre-incubation as well as incubation times were the same as those used in perfusion. Uptake was determined from the net radioactivity of 2-deoxyglucose and its phosphate ester contained by the muscle. Glucose uptake (R'g) by both muscles was not affected by 10 μM 5-HT. Insulin (15 nM) significantly ($P < 0.001$, unpaired Student's t test) increased the rate of glucose uptake by 115% (soleus) and 154% (extensor digitorum longus). Even when added before insulin 5-HT had no significant effect on the insulin-mediated stimulation of glucose uptake in either muscle.

5.3.2 *Experiments involving NE at 37°C.*

At 37°C, NE-mediated effects on perfusion pressure and $\dot{V}O_2$ were qualitatively similar to those observed at 25°C (Chapter 2, Dora *et al.*, 1992). NE dose-dependently increased perfusion pressure, and at low doses, mediated increases in $\dot{V}O_2$, but at higher doses inhibited $\dot{V}O_2$ at 37°C (Figure 5.6). The effects on $\dot{V}O_2$ and pressure were totally reversible upon removal of NE. In perfusions performed at 25°C, decreasing the size of perfused rats from 180-200 g to 70-80 g (with the same relative flow rate per gram tissue) shifted the NE dose response curve for $\dot{V}O_2$ and perfusion pressure to the right (Figure 5.6). Using 70-80 g rats, increasing the flow rate shifted the dose response curve to the left, and, at this high flow rate, increasing the perfusion temperature to 37°C shifted the dose curve to the right and increased the maximal $\dot{V}O_2$ response. At 37°C, 1 μM NE induced an almost maximal rise in $\dot{V}O_2$ in association with increased perfusion pressure, and 10 μM NE inhibited $\dot{V}O_2$ to values below basal in association with further rises in perfusion pressure (Figure 5.6). 1 μM and 10 μM NE were used in this study to mediate stimulatory and inhibitory effects on $\dot{V}O_2$, respectively.

Mean values ± SE for $\dot{V}O_2$, perfusion pressure, lactate release and glucose uptake obtained 50 min after commencement of treatment (at 90 min) are given in Figure 5.11. In control perfusions, $\dot{V}O_2$, perfusion pressure, lactate release and

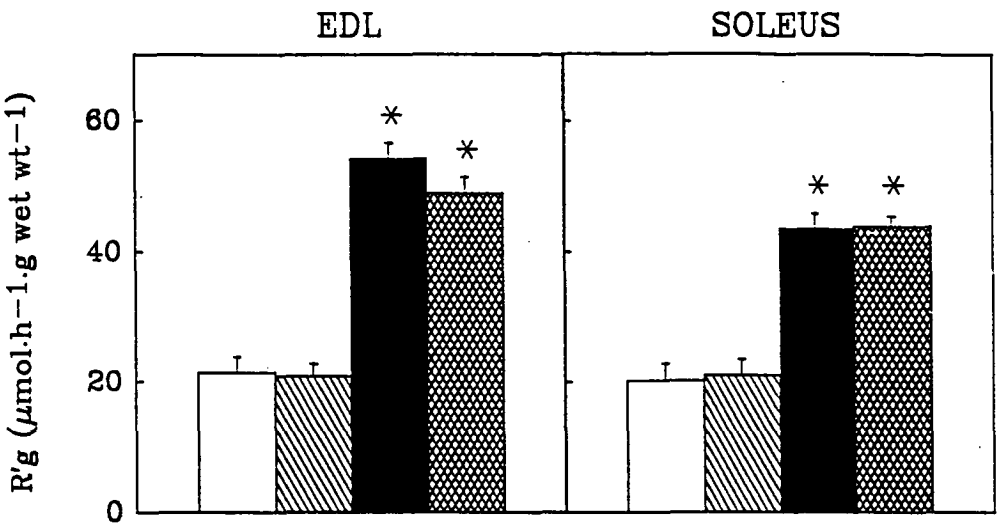

Figure 5.5 Effects of 5-HT, insulin and 5-HT plus insulin on glucose uptake by isolated incubated soleus and EDL muscles.

Muscles (20-30 mg) were incubated with medium identical to that used for perfusions. After a preincubation period of 40 min, muscles were transferred to fresh medium and vehicle (□), 10 μM 5-HT (▨), 15 nM insulin (■) or 10 μM 5-HT + 15 nM insulin (▩) were added. 2-Deoxy-D-[1-^{3}H]glucose and [U-^{14}C]sucrose were added after a further 20 min and the incubations continued for a total of 90 min. Muscles were analysed for radioactivity as described in Methods. Values shown are means ± SE for 8 muscles (both limbs from four rats in each group). *$P < 0.001$ significantly different from vehicle. There was no significant difference between vehicle and 5-HT or between insulin and insulin + 5-HT. 5-HT, serotonin; EDL, extensor digitorum longus.

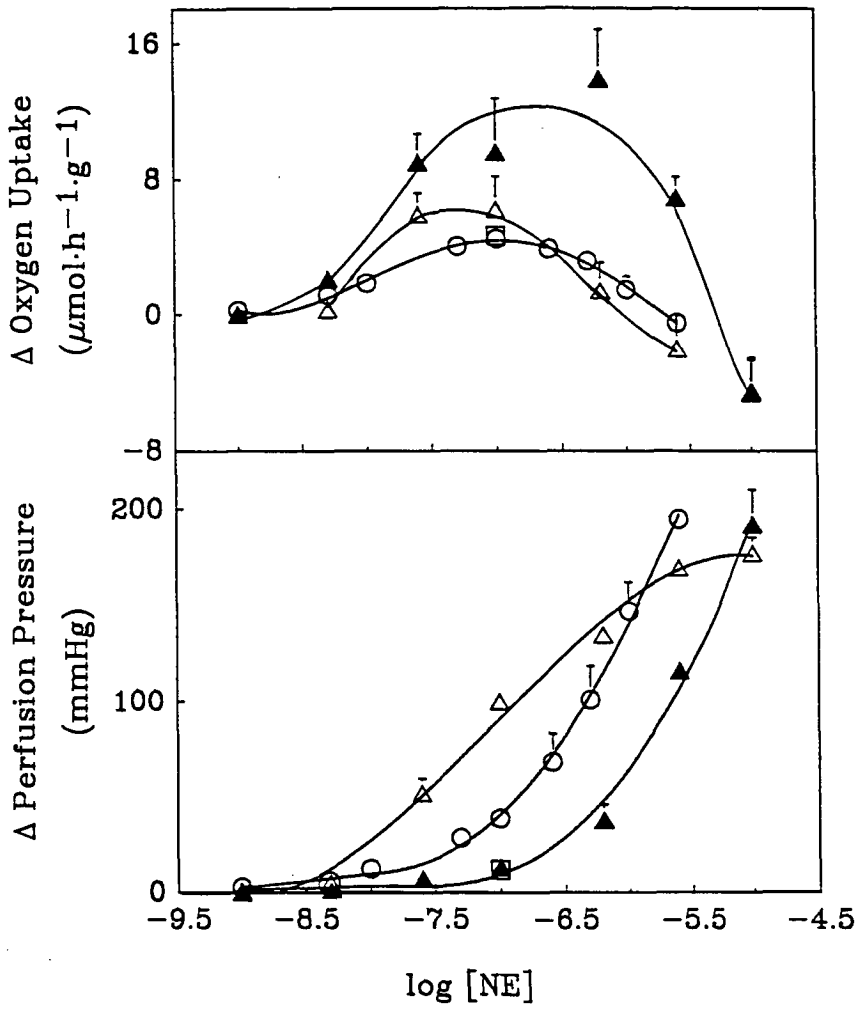

Figure 5.6 **Effects of perfusion temperature, flow rate and rat size on the dose-dependent $\dot{V}O_2$ and perfusion pressure effects of NE in hindlimbs.**

Basal perfusion conditions are given in Table 5.1. Each dose of NE was infused constantly for 10 min into either one hindlimb of 180-200 g rats perfused at 4 ml.min^{-1} and 25°C (○, n = 5, from Chapter 2); or two hindlimbs of 70-80 g rats perfused at 3 ml.min^{-1} and 25°C (□, n = 4), 8 ml.min^{-1} and 25°C (△, n = 2), or 8 ml.min^{-1} and 37°C (▲, n = 2). Values are means ± SE; n, no. of hindlimb perfusions; NE, norepinephrine; $\dot{V}O_2$, oxygen uptake.

glucose uptake remained constant for the 90 min experimental period (Figures 5.7 & 5.8).

5.3.2.1 Effects of insulin in perfused hindlimbs.

Insulin (15 nM) had little effect on perfusion pressure, but caused a small increase in $\dot{V}O_2$ (7.0%) that was apparent 10 min after addition (Figure 5.7, left panels). Insulin markedly stimulated lactate release and glucose uptake to steady-state effects 1.5-fold & 2.7-fold, respectively, above corresponding controls (Figure 5.8, left panels). These effects of insulin were of similar magnitude to those observed at 32°C in larger rats.

5.3.2.2 Effects of low-dose NE in perfused hindlimbs.

Low-dose NE (1 μM) caused a marked increase in $\dot{V}O_2$ (1.5-fold) in association with vasoconstriction and these effects were maintained until the end of the perfusion (Figure 5.7, left panels). Lactate release was rapidly and significantly ($P < 0.001$, unpaired Student's t test) increased to a peak value (2.1-fold) which returned to steady state levels above basal (1.4-fold) (Figure 5.8, upper left panel), qualitatively consistent with results at 25°C (Chapter 2, Hettiarachchi *et al.*, 1992). Glucose uptake was also transiently and significantly ($P < 0.05$, unpaired Student's t test) increased (1.5-fold) and returned to steady-state levels approaching basal (1.2-fold) within 10 min (Figure 5.8, lower left panel).

5.3.2.3 Effects of insulin plus low-dose NE in perfused hindlimbs.

Insulin appeared to have a vasodilatory effect against low-dose NE (1 μM), as perfusion pressure was significantly reduced compared to NE alone (Figure 5.7, bottom panels, Figure 5.11). Insulin- and NE-mediated lactate release were additive and NE attenuated the insulin-stimulated glucose uptake (Figures 5.8 and 5.11).

The effects of low-dose NE plus insulin on $\dot{V}O_2$ and perfusion pressure were augmented by the β-adrenoceptor (AR) antagonist propranolol (10 μM) and totally inhibited by the α_1-AR antagonist prazosin (2.5 μM) (Figure 5.7, right panels). Propranolol, but not prazosin, reduced the NE plus insulin-mediated steady state lactate release without affecting the initial peak. Prazosin inhibited the NE-stimulated

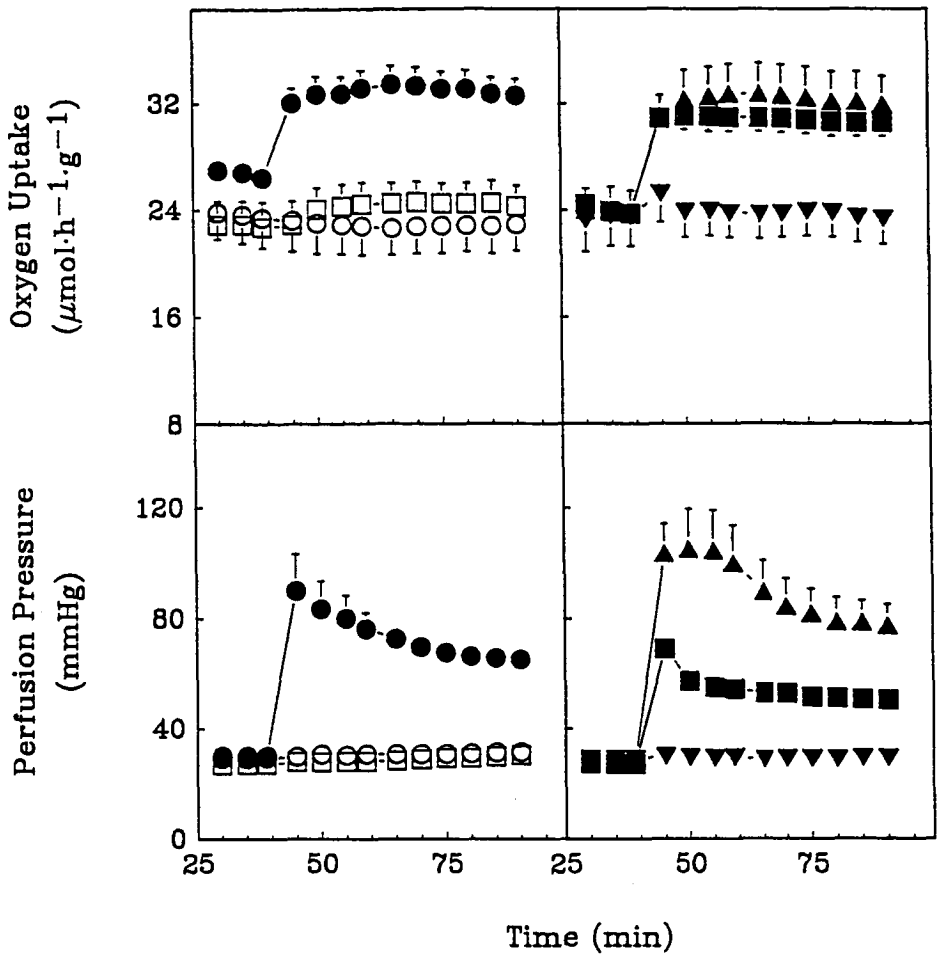

Figure 5.7 Effects of insulin, and low-dose NE in the presence and absence of insulin and AR antagonists, on hindlimb $\dot{V}O_2$ and perfusion pressure.

Hindlimbs from 70-80 g rats were perfused at a constant flow rate of 8 ml.min^{-1} at 37°C in a non-recirculating mode as described in Methods. The following constant infusions were commenced at 40 min to give the final concentrations shown and these were maintained until the end of perfusion: no addition (○); 15 nM insulin (□); 1 μM NE (●); 1 μM NE + 15 nM insulin (■); 1 μM NE + 15 nM insulin + 10 μM (±)-propranolol (▲); 1 μM NE + 15 nM insulin + 2.5 μM prazosin (▼). Values are means ± SE for 4 perfusions. When not visible, error bars are within symbols. AR, adrenoceptor; NE, norepinephrine; $\dot{V}O_2$, oxygen uptake.

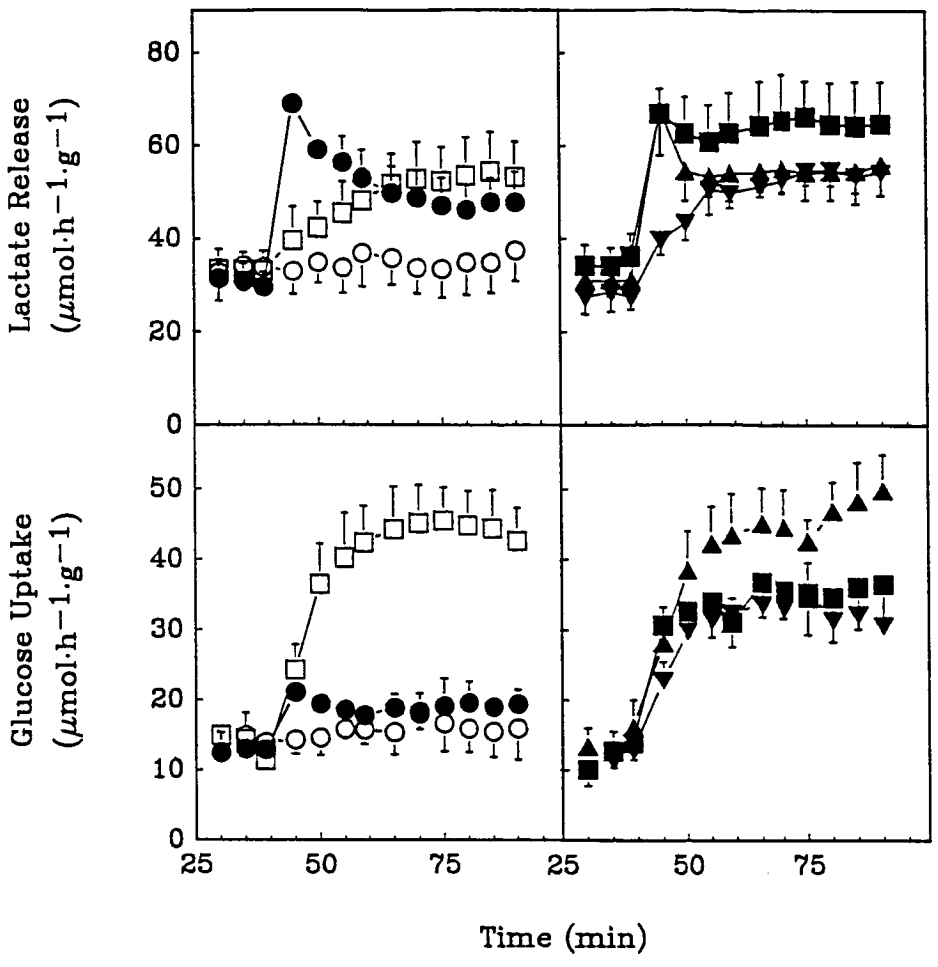

Figure 5.8 Effects of insulin, and low-dose NE in the presence and absence of insulin and AR antagonists, on hindlimb lactate release and glucose uptake.

Hindlimbs were perfused, and additions were made, as described in Figure 5.2. The following constant infusions were commenced at 40 min to give the final concentrations shown and these were maintained until the end of perfusion: no addition (○); 15 nM insulin (□); 1 μM NE (●); 1 μM NE + 15 nM insulin (■); 1 μM NE + 15 nM insulin + 10 μM (±)-propranolol (▲); 1 μM NE + 15 nM insulin + 2.5 μM prazosin (▼). Values are means ± SE for 4 perfusions. When not visible, error bars are within symbols. AR, adrenoceptor; NE, norepinephrine.

peak in lactate release. Propranolol, but not prazosin, reversed the NE-mediated inhibition of insulin-stimulated glucose uptake (Figure 5.8, right panels).

5.3.2.4 Effects of high-dose NE in perfused hindlimbs.

High-dose NE (10 μM) stimulated prazosin-sensitive increases in perfusion pressure associated with inhibition (32% below basal) of $\dot{V}O_2$ (Figure 5.9). $\dot{V}O_2$ was transiently increased during the rise in perfusion pressure (data not shown), but after 5 min of infusion had already fallen to levels below basal. Lactate release was markedly reduced compared to that observed upon addition of low-dose NE, although the steady state level after 20 min was elevated. High-dose NE had no effect on glucose uptake (Figure 5.10, left panels).

5.3.2.5 Effects of insulin plus high-dose NE in perfused hindlimbs.

Insulin slightly attenuated (7.1%) the high-dose NE-mediated rise in perfusion pressure and inhibition of $\dot{V}O_2$ (Figure 5.9, right panels). High-dose NE plus insulin was not additive on lactate release, and insulin-stimulated glucose uptake was markedly and significantly impaired in the presence of NE (Figure 5.10, right panels, Figure 5.11).

Propranolol augmented the NE plus insulin-mediated effects on $\dot{V}O_2$ and perfusion pressure, and the combination of prazosin plus propranolol almost totally blocked the rise in perfusion pressure, but stimulated $\dot{V}O_2$ (Figure 5.9, right panels). This effect was similar to that observed upon infusion of approx. 25 nM NE (Figure 5.1), and was consistent with a β-AR-mediated vasodilatation of the vasoconstriction associated with increases in $\dot{V}O_2$ (Chapter 2, Colquhoun *et al.*, 1990). Higher doses of prazosin abolished the stimulation of $\dot{V}O_2$ and perfusion pressure (data not shown).

Propranolol totally opposed the high-dose NE and insulin-stimulated lactate release despite augmenting the effects on $\dot{V}O_2$ and pressure. Propranolol slightly attenuated the NE-mediated inhibition of insulin-stimulated glucose uptake (Figure 5.10, right panels).

Prazosin markedly attenuated the high-dose NE-mediated inhibition of lactate release, and demonstrated that β-AR-stimulation inhibited insulin-mediated glucose uptake (Figure 5.10 right panels).

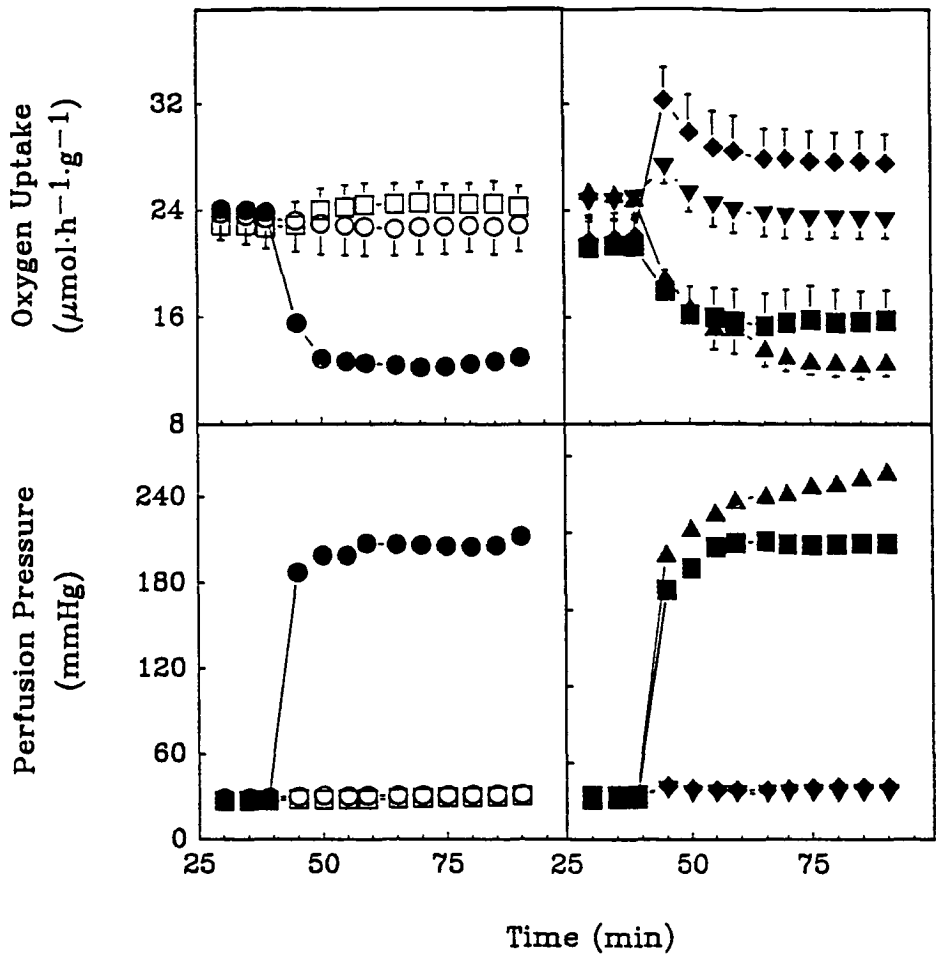

Figure 5.9 Effects of insulin, and high-dose NE in the presence and absence of insulin and AR antagonists, on hindlimb $\dot{V}O_2$ and perfusion pressure.

Hindlimbs were perfused, and additions made as described in Figure 5.2, except 10 μM NE was used. The following constant infusions were commenced at 40 min to give the final concentrations shown and these were maintained until the end of perfusion: no addition (○); 15 nM insulin (□); 10 μM NE (●); 10 μM NE + 15 nM insulin (■); 10 μM NE + 15 nM insulin + 10 μM (±)-propranolol (▲); 10 μM NE + 15 nM insulin + 2.5 μM prazosin (▼); 10 μM NE + 15 nM insulin + 10 μM (±)-propranolol + 2.5 μM prazosin (◆). Values are means ± SE for 4 perfusions. When not visible, error bars are within symbols. AR, adrenoceptor; NE, norepinephrine; $\dot{V}O_2$, oxygen uptake.

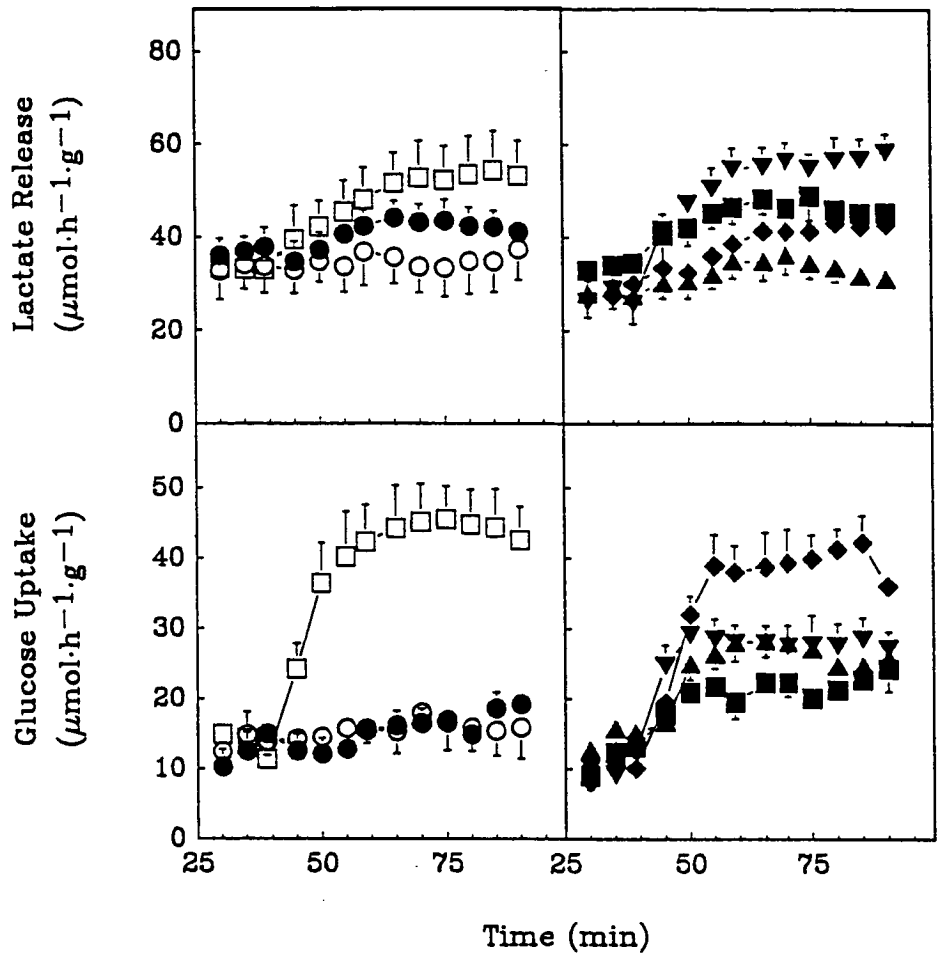

Figure 5.10 Effects of insulin, and high-dose NE in the presence and absence of insulin and adrenoceptor antagonists, on hindlimb lactate release and glucose uptake.

Hindlimbs were perfused and additions made as described in Figure 5.4. The following constant infusions were commenced at 40 min to give the final concentrations shown and these were maintained until the end of perfusion: no addition (O); 15 nM insulin (□); 10 μM NE (●); 10 μM NE + 15 nM insulin (■); 10 μM NE + 15 nM insulin + 10 μM (±)-propranolol (▲); 10 μM NE + 15 nM insulin + 2.5 μM prazosin (▼); 10 μM NE + 15 nM insulin + 10 μM (±)-propranolol + 2.5 μM prazosin (◆). Values are means ± SE for 4 perfusions. When not visible, error bars are within symbols. AR, adrenoceptor; NE, norepinephrine.

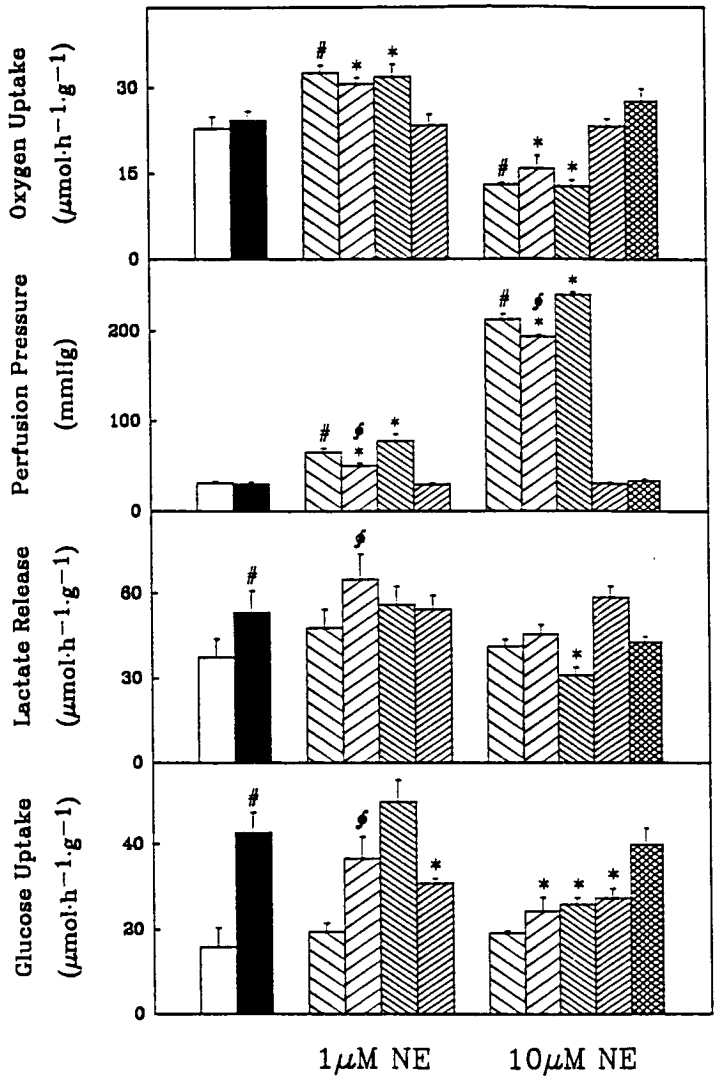

Figure 5.11 Average steady-state effects of insulin, and NE in the presence and absence of insulin and AR antagonists, on hindlimb $\dot{V}O_2$, perfusion pressure, lactate release and glucose uptake.

Values obtained 50 min after commencement of treatment (at 90 min) from Figures 5.2-Figure 5.5 were averaged for control (□); 15 nM insulin (■); NE (▧); NE + 15 nM insulin (▨); NE + 15 nM insulin + 10 μM (±)-propranolol (▩); NE + 15 nM insulin + 2.5 μM prazosin (▨); NE + 15 nM insulin + 10 μM (±)-propranolol + 2.5 μM prazosin (■). Values are means ± SE of four perfusions for each treatment. Significance of differences ($P < 0.05$) assessed using one way ANOVA: #, control different from insulin or NE; *, insulin different from insulin + NE ± antagonist combinations; §, NE different from insulin + NE. AR, adrenoceptor; NE, norepinephrine; $\dot{V}O_2$, oxygen uptake.

The combination of β- and α_1-AR blockade slightly attenuated (not significant) the insulin-stimulated lactate release and glucose uptake (Figure 5.10). Interestingly, no peak in lactate release was observed upon addition of NE despite the increases in $\dot{V}O_2$, suggesting α_1-AR lactate release is associated with vasoconstriction.

Mean values ± SE for $\dot{V}O_2$, perfusion pressure, lactate release and glucose uptake obtained 50 min after commencement of treatment (at 90 min) are given in Figure 5.11.

5.4 Discussion.

The present study confirms the previous findings of β-AR and insulin stimulated increases in hindlimb glucose uptake and lactate release (Chiasson *et al.*, 1981; Richter *et al.*, 1982). It also confirms the low dose NE α_1-AR stimulated increases in $\dot{V}O_2$ and lactate release (Chapter 2) and high dose NE α_1-AR-stimulated and 5-HT_{2A}-stimulated inhibition of hindlimb $\dot{V}O_2$ and lactate release (Chapter 2, Dora *et al.*, 1991, 1992). These results were qualitatively similar despite quantitative changes when perfusion medium, temperature and flow rate and rat size were altered[1]. Also of interest was the lack of appreciable effect by low-dose NE on hindlimb glucose uptake, remaining consistent with a lack of α_1-AR mediated glucose uptake proposed by Chiasson *et al.* (1981) but not Richter *et al.* (1982a).

In general, the basal and maximum insulin-stimulated rate of glucose uptake compared well with rates obtained by other workers (Chiasson *et al.*, 1981; Kubo & Foley, 1986; Klip *et al.*, 1987; Richter *et al.*, 1988), whether erythrocytes were or were not included in the perfusion medium (Table 5.2).

Evidence was also obtained supporting the vascular effects of supraphysiological doses of insulin. 15 nM insulin vasodilated against both low and

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1. One striking feature of the NE response in small rats was the large increases in $\dot{V}O_2$ associated with only small rises in perfusion pressure. This may have been due to different flow patterns through the hindlimb of younger rats. In hamster cremaster muscle, Sarelius *et al.* (1981) observed that age significantly modified microvascular parameters related to tissue oxygen supply. The microcirculation in juvenile hamsters was characterized by small intercapillary distances, short capillary lengths and tortuous vessels (Sarelius *et al.*, 1981).

Rat Size (g)	Flow Rate ($ml.min^{-1}$)	Temp (°C)	RBC (+/-)	Glucose Uptake ($\mu mol.h^{-1}.g^{-1}$)		[Insulin] (mU/ml)	*n*
				Basal	Insulin		
240-270	8	25	-	1.4 ± 0.2	4.9 ± 0.1	10.0	2
240-270	15	32	-	9.8 ± 0.5	24.4 ± 0.7	2.0	4
70-80	8	37	-	16.5 ± 1.2	44.8 ± 1.8	2.0	4
170-230	10	37	+	1.0 ± 0.6	14.6 ± 1.2	12.5	6[A]
180-220	10	37	+	5.5 ± 1.0	19.3 ± 3.3*	1.0	5[B]
200-230	8	37	#	12	25*	14.0	4[C]
250-350	20	37	-	8.0 ± 0.8	42.0 ± 3.8*	1.0	15[D]

Table 5.2 Effect of insulin on perfused hindlimb glucose uptake.

Rats were perfused with constant flow at different temperatures (Temp) with or without erythrocytes (RBC) in the perfusion medium. Values are means ± SE; *n*, no. of hindlimbs; A, from Ruderman *et al.*, 1971; B, from Chiasson *et al.*, 1981; C, from Kubo & Foley, 1986; D, from Klip *et al.*, 1987; *, using average rat body mass and assuming 8.3% of body mass is skeletal muscle (Ruderman *et al.*, 1971); #, artificial erythrocyte substitute.

high doses of NE, but not against 5-HT. This is consistent with reports of vasodilatory actions of insulin against NE in rat tail artery (Alexander *et al.*, 1977), and against NE and 5-HT in perfused rat mesenteric artery (Wambach & Liu, 1992). In contrast, however, in the perfused rat mesenteric arcade, Townsend *et al.* (1992) found insulin augmented the NE-mediated (but not 5-HT-mediated) rises in pressure. Wambach and Liu (1992) concluded the vasodilatory action of insulin was not receptor-specific, and noted insulin could affect the activity of the Na^+/K^+-ATPase, the Na^+/H^+ antiporter and the Na^+/Ca^{2+} antiporter (Ferrari & Weidmann, 1990).

The present study exemplified the importance of flow distribution on hindlimb metabolism. The consistent theme was an inhibition of hindlimb metabolic effects in situations where it is proposed flow was being limited to muscle. It reports for the first time a marked high-dose NE- and 5-HT-mediated inhibition of insulin-mediated glucose uptake and lactate release in the constant flow perfused rat hindlimb that was not apparent in unperfused incubated muscles. The present findings are consistent with our previous observations (Dora *et al.*, 1991, 1992) where high-dose NE and 5-HT caused a marked inhibition of $\dot{V}O_2$ concomitant with vasoconstriction in the perfused hindlimb. The proposed explanation to account for the past (Dora *et al.*, 1991, 1992) and present data focuses on the notion of vascular shunting in the hindlimb in which a subgroup of prazosin-sensitive α_1-ARs with relatively low affinity for NE (Chapter 2) and 5-HT_{2A} receptors mediate a marked constriction in large arterioles down stream from branch points for functional shunts leading to the venous circulation (Chapter 7). If this is the case, then complete or near-complete closure at these points would effectively return perfusate to the venous circulation, allowing constant flow, whilst preventing access of the perfusate to significant areas of the microvasculature. Such effects would not be evident when unperfused muscle is incubated (Sasson *et al.*, 1990) but would markedly affect nutrient delivery in perfused hindlimb. Thus sites where other vasoconstrictors such as norepinephrine, vasopressin and angiotensin II mediate increased $\dot{V}O_2$ (Colquhoun *et al.*, 1988) may be compromised by diminished supply of O_2 resulting in a marked inhibition of $\dot{V}O_2$.

The occurrence of vascular shunting in skeletal muscle *in vivo* has profound implications for nutrient and hormone delivery as well as product removal and raises many questions concerning the influences that may operate to affect its control. In addition vascular shunting mediated by site-specific vasoconstrictors, such as NE and 5-HT in the present study, may have implications for diabetes. There is growing interest in the relationship between hypertension and insulin resistance in both humans

and experimental animals. Indeed, the combination of glucose intolerance and hyperinsulinaemia seen in many hypertensive humans (Shen *et al.*, 1988; reviewed by Julius *et al.*, 1992), points to a defect in insulin-stimulated glucose uptake. Similarly, animal models such as the spontaneously hypertensive rat (SHR) (Mondon & Reaven, 1988; Finch *et al.*, 1990) and fructose-fed rat (Hwang *et al.*, 1987) appear to be insulin resistant as well as hypertensive. However it is not clear whether the link between hypertension and insulin resistance is indicative of a causal relationship. For humans, the lowering of high blood pressure with antihypertensive drugs does not necessarily decrease plasma insulin concentration to normal. In some instances treatment of the hypertension accentuates the degree of hyperinsulinaemia (Sowers, 1991). In others, particularly those treated with either the angiotensin-converting enzyme inhibitor, captopril (Pollare *et al.*, 1989), the α_1-AR antagonist, prazosin (Pollare *et al.*, 1988; Swislocki *et al.*, 1989) or the 5-HT_{2A} antagonist, ketanserin (Janka *et al.*, 1988; reviewed in Janssens *et al.*, 1991), peripheral blood flow and insulin sensitivity may improve. The ability of prazosin and ketanserin to preferentially oppose the α_1-AR subgroup mediating an inhibition of $\dot{V}O_2$ (Chapter 2, Figure 2.9) remains consistent with an improvement of insulin access to muscle. Thus whilst hypertension in insulin-resistant states has generally been attributed to hyperinsulinaemia with its enhanced sympathetic nervous system activation (Landsberg & Krieger, 1989), the relationship between cause and effect remains unclear for humans as well as animal models (Mondon & Reaven, 1988; Finch *et al.*, 1990; Buchanan *et al.*, 1992; Bursztyn *et al.*, 1992). From the previous (Dora *et al.*, 1991, 1992) and present studies, the action of 5-HT on the perfused hindlimb has provided a useful model.

However 5-HT may not be a physiological regulator of skeletal muscle vascular shunts *in vivo*, as circulating levels are low due to rapid uptake into platelets and other tissues (Bhat & Block, 1990). However, Barradas *et al.* (1988) found that circulating 5-HT levels were almost 3 fold greater in patients with NIDDM, presumably due to 5-HT release from platelets. In these latter patients, the 5-HT concentration range was between 0.1 and 1 μM (Barradas *et al.*, 1988). There is also some evidence that 5-HT may be taken up into sympathetic nerve endings (Verbeuren *et al.*, 1983; Kawasaki & Takasaki, 1984; Urabe *et al.*, 1991). Thus with the possibility of high local concentrations, the effects observed in this study may have physiological relevance. There have been numerous reports that *in vivo* administration of 5-hydroxytryptophan, the immediate precursor of 5-HT, produces

hypoglycaemia in a variety of animal models and in general these effects are blocked by 5-HT antagonists (see Wozniak & Linnoila, 1991, and references therein). The subtype of receptors involved in the hypoglycaemic response appears to be 5-HT_1 and therefore may be different from the 5-HT_{2A} receptors involved in shunting in the hindlimb. In addition, the systemic administration of 5-HT receptor agonists can alter the neurochemical systems known to regulate the sympathetic nervous system. Thus a recent report (Chaouloff *et al.*, 1990) that the 5-HT_2 receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane triggers a dose dependent increase in plasma glucose was concluded to be attributable to an intermediary involvement of catecholamines. The hyperglycaemic and hyperinsulinaemic effect of another 5-HT_2 agonist, α-methyl serotonin (Chaouloff *et al.*, 1990) did not appear to be a central nervous system-mediated effect and could not be explained.

Another controlling influence for vascular shunts may be exercise where local vasodilatation (Armstrong & Laughlin, 1983) and changes in blood flow have been demonstrated (Harrison *et al.*, 1990a). This relationship is investigated in Chapter 6.

Recently we have noted that high concentrations of norepinephrine can also result in apparent shunting in the perfused hindlimb (Dora *et al.*, 1992). Thus it is possible that a major controlling mechanism involves sympathetic vasoconstrictor nerves (Iversen & Nicolaysen, 1990). From perfusion experiments on the isolated dog hindleg and on dog gastrocnemius, Pappenheimer (1941) proposed that reduced $\dot{V}O_2$ was the result of sympathetic nerves acting on blood vessels to divert blood flow from capillaries to arteriovenous anastomoses. Durán and Renkin (1976) investigated this phenomenon in some detail, and although their conclusion differed from Pappenheimer's, they found that high frequency (8-16 Hz) supramaximal stimulation of the lumbar trunk led to an inhibition of $\dot{V}O_2$ with a rise in resistance, but low frequency (0.5-4 Hz) stimulated increased $\dot{V}O_2$ as well as resistance (Durán & Renkin, 1976). Intermediate frequency (4-8 Hz) stimulation was often associated with an initial fall followed by an increase in $\dot{V}O_2$ and relatively constant resistance (Durán & Renkin, 1976). The observations in this study where low and high concentrations of norepinephrine stimulate then inhibit $\dot{V}O_2$ respectively, during vasoconstriction of the hindlimb (Colquhoun *et al.*, 1988; Dora *et al.*, 1992) appear to be consistent with the nerve stimulation studies (Pappenheimer, 1941; Durán & Renkin, 1976). Thus it is possible that increased sympathetic nerve stimulation may also impair insulin-mediated glucose uptake.

To summarize, both 5-HT and high doses of NE, but not low doses of NE, cause marked acute insulin resistance in perfused hindlimb muscle that is not apparent when isolated muscles are incubated with 5-HT *in vitro*. It is proposed that the acute insulin resistance results from site-specific vasoconstriction on large arterioles to cause vascular shunting and diminished access by both insulin and glucose. Whether the findings have implications for the association between hypertension and insulin resistant diabetes will depend on whether shunting occurs *in vivo* and the prevailing regulatory mechanisms.

CHAPTER 6

Effect of 5-HT during skeletal muscle contraction.

6.1 Introduction.

Infusion of serotonin (5-HT) into the constant-flow perfused rat hindlimb causes a marked decrease in oxygen uptake ($\dot{V}O_2$) and insulin-mediated glucose uptake (Chapters 2-5). These findings are consistent with the hypothesis of functional vascular shunting in the hindlimb where 5-HT mediates a marked constriction in large arteries, causing a redistribution of flow within the hindlimb. 5-HT-mediated complete or near complete closure at these points would effectively return perfusate to the venous circulation, allowing constant flow, whilst preventing or reducing access of the perfusate to areas of the microvasculature. Such effects would seriously impair nutrient delivery in perfused hindlimb, but would not be expected to be apparent when unperfused muscle was incubated.

Several studies have shown that changes in blood flow can alter the force exerted by a muscle contracting intermittently during an extended period. This has been demonstrated in normal man (Barcroft & Millen, 1939), in man with cardiac impairment (Donald *et al.*, 1957) and in the isolated cat gastrocnemius-soleus (Hirvonen & Sonnenschein, 1962; Wright & Sonnenschein 1965). The communication among endothelial and smooth muscle cells normally causes vasodilatation and thereby hyperaemia to the exercising muscle by a combination of flow-dependent and endothelial cell-mediated relaxation (reviewed in Segal, 1992). Evidence suggests that the locus of flow control shifts from the microvessels up the resistance network by an as yet undetermined method of cell-to-cell communication involving the endothelium (Segal & Duling, 1986; Segal *et al.*, 1989), to encompass the feed arteries, which are external to the muscle (Hilton, 1959; Folkow *et al.*, 1971; Segal & Duling, 1986).

However, in addition to vasodilatation, exercise *in vivo* also increases sympathetic nervous system activity, predominantly to redistribute cardiac output away from inactive areas. As exercise intensity increases, the maximal flow to contracting muscle can be limited by sympathetic vasoconstriction (Joyner *et al.*, 1990, 1992), apparently at the level of the feed arteries external to the active muscle

(Lind & Williams, 1979). The vasoconstriction overcomes the dilatation of the feed arteries (Folkow *et al.*, 1971), thus suggesting diminished effectiveness of upstream communication.

It is unknown whether upstream communication occurs in the constant-flow, perfused rat hindlimb preparation used in these studies. However, using the same perfusion system, exercise-stimulated vasodilatation decreased the vasoconstriction by angiotensin II (Colquhoun *et al.*, 1990).

In the present study the effect of 5-HT on tension development and contraction-induced increase in $\dot{V}O_2$ by the electrically stimulated gastrocnemius-plantaris-soleus muscle group in the constant-flow perfused rat hindlimb (with and without erythrocytes at 37 and 25°C, respectively) was examined. Also the effect of contraction-induced vasodilatation on 5-HT-mediated vasoconstriction was investigated. These results were compared to the effects of 5-HT on tension development by isolated, incubated muscles.

6.2 Materials and methods.

6.2.1 Perfused hindlimbs.

Surgery was performed on 180-200 g rats as outlined in Section 2.2.2.1.2 with the following alterations and additions. After cannulation and commencement of perfusion the rat was placed ventral side down. The skin was removed from the thigh of the left hindlimb, the sciatic nerve was exposed in the flank and cut to allow the positioning of the distal cut end in a suction electrode. The knee was secured by the tibiopatellar ligament and the Achilles tendon was attached to a Harvard Apparatus isometric transducer (Figure 6.1).

Perfusions were performed in a temperature-controlled cabinet, and arterial perfusion medium was temperature-equilibrated by passage through an in-line heat exchanger and water-jacketed arterial line. In addition, the oxygen electrode was water-jacketed. In preliminary experiments, in-line thermistors monitored perfusion temperatures.

The perfusate samples for net lactate output determination were taken at 2 min intervals before and during commencement of treatments. The rates of lactate release and $\dot{V}O_2$ were calculated from arteriovenous difference and flow rate as described in Chapter 2 (Section 2.2).

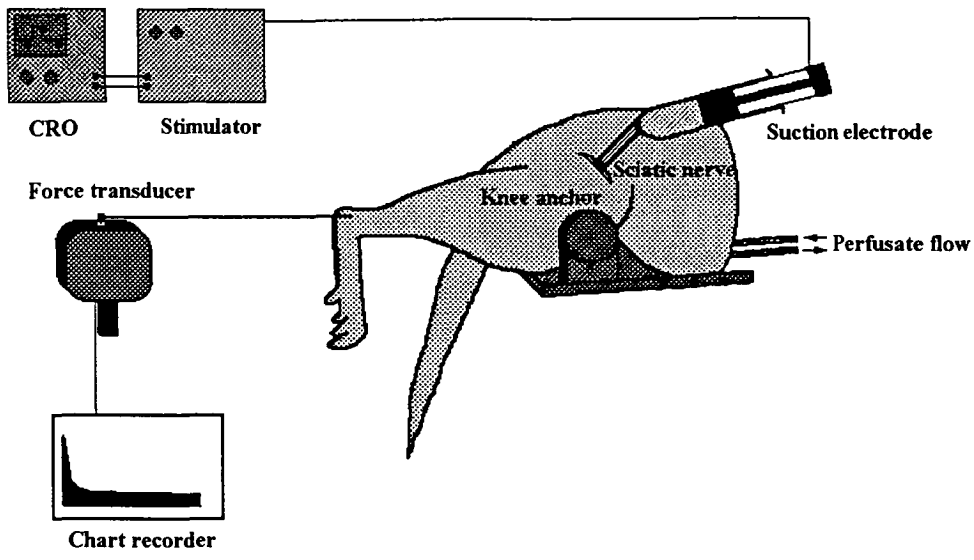

Figure 6.1 Apparatus used to electrically stimulate and measure tension development in the GPS muscle group.

180-200 g rats were placed dorsal side down temperature controlled conditions and perfused at a constant flow rate (for perfusion apparatus see Chapter 2, Figure 2.3). The knee was held in position and tension development in the GPS muscle group was monitored through the Achilles tendon. The lower hindlimb muscle groups were stimulated via the sciatic nerve. 5-HT or its vehicle were infused into the arterial line immediately prior to a small bubble trap/mixing chamber. GPS, gastrocnemius-plantaris-soleus; 5-HT, serotonin; CRO, cathode ray oscilloscope.

Arterial perfusion pressure was corrected for the pressure fall due to the resistance of the cannula.

6.2.1.1 Erythrocyte-free perfusions.

The perfusions were performed at 25°C using modified Krebs-Henseleit buffer containing 8.3 mM glucose and 1.27 mM $CaCl_2$ with 2% bovine serum albumin (BSA). The buffer reservoir was gassed with 95% O_2-5% CO_2 at 4°C to enable full oxygenation. At equilibration (after 30 min perfusion at 4 $ml.min^{-1}$) and prior to electrical stimulation, the flow rate was increased to 17.0 ± 0.2 $ml.min^{-1}$. The resting length of the muscle was adjusted to attain maximum active tension upon stimulation (this normally required 4 or 5 tetanic stimulations). After attainment of a new steady state for $\dot{V}O_2$ and perfusion pressure (10 min at the higher flow rate), electrical stimulation was commenced using 200 ms trains of 100 Hz 0.1 ms pulses applied every 2 s and adjusted (3-5 V) to attain full fibre recruitment. Tension development transmitted through the Achilles tendon, perfusion pressure and venous PO_2 were recorded continuously, and effluent perfusate samples were regularly collected for lactate analysis.

Electrical stimulation of the sciatic nerve contracted approx. 30% of the perfused hindlimb mass, including the gastrocnemius, soleus, and plantaris muscle groups, hamstring muscles, deep portion of the crural muscles, and the lateral crural muscles (Rennie & Holloszy, 1977). Thus during the experiments, changes in perfusion pressure and metabolism were due to a combination of contracting lower-limb and uncontracting upper-hindlimb tissue. Attempts at selectively perfusing the contracting mass via the femoral artery and vein resulted in collateral circulation to the upper hindlimb, thus making determination of lower leg metabolism inaccurate.

6.2.1.1.1 Effect of prior infusion of 5-HT on skeletal muscle contraction.

5-HT (0.25 μM) was continuously infused for 6 min prior to and throughout sciatic nerve stimulation. This allowed assessment of steady-state 5-HT induced effects, and both the effects of 5-HT on contraction and *vice versa*.

6.2.1.1.2 Effect of 5-HT during skeletal muscle contraction.

5-HT (0.25 μM) was infused for a brief 2 min period during the 20 min period of contraction-stimulation. , This allowed determination of the reversibility of the 5-HT effects during contraction.

6.2.1.2 Erythrocyte perfusions.

For perfusions at 37°C, fresh bovine erythrocytes were washed 3 times using saline (0.9% NaCl), and 3 times using Krebs-Henseleit buffer and were added to modified Krebs-Henseleit medium containing 2.5 mM $CaCl_2$ and 4% BSA (35% haematocrit). Perfusion was initially commenced at 4 $ml.min^{-1}$ for 30 min until equilibration and then the flow rate increased to 15.3 ± 0.2 $ml.min^{-1}$. As for the erythrocyte-free perfusions, the resting length of the muscles were then adjusted to attain maximum active tension. Samples of venous perfusate were collected in gas-tight syringes at basal conditions (approx. 2 min before electrical stimulation), 4 min after stimulation (before infusions) when plateau tension development was reached, 3 min after vehicle or 5-HT infusion (when maximum changes due to 5-HT were attained), and 19 min after commencement of electrical stimulation. To determine the times for collecting these samples a continuous qualitative assessment of venous effluent oxygen content was made during initial perfusions using an in-line Clark-type oxygen electrode. Arterial samples were taken from an in-line arterial septum at basal conditions and at the completion of the perfusion which corresponded to approx. 25 min after commencement of electrical stimulation. Samples of perfusate were analyzed for total oxygen content using a galvanic cell oxygen analyser (TasCon Oxygen Content Analyser, manufactured by the Physiology Department, University of Tasmania). Duplicate analysis of each collected sample took an average of 15 min.

From estimates conducted by others (Ruderman *et al.*, 1980) of 0.4 μmole lactate $produced.ml^{-1}.h^{-1}$ bovine erythrocytes and an estimated passage time of 1 min for the perfusion media through the apparatus and hindlimb at 15.3 ± 0.2 $ml.min^{-1}$, it was calculated that the erythrocytes would contribute only 0.5 $\mu mol.h^{-1}.g^{-1}$. This small contribution to lactate release by erythrocytes was ignored.

6.2.2 *Incubated muscles.*

Male hooded Wistar rats (60-70 g) were anaesthetized with an *intraperitoneal* injection of aqueous pentobarbital sodium (6-8 mg/100 g body weight) containing heparin (100 U/100 g body wt). The soleus and extensor digitorum longus (EDL) muscles from each leg were quickly and carefully removed and placed in 95% O_2-5% CO_2 gassed incubation buffer until use.

A modified Krebs-Henseleit buffer was used which consisted of (mM): 118 NaCl, 4.74 KCl, 1.19 KH_2PO_4, 1.18 $MgSO_4$, 25 $NaHCO_3$, 1.27 $CaCl_2$, 5 glucose, 5 pyruvate, 35 mannitol and 5 HEPES. After gassing, the pH was 7.35 at 25°C.

Incubations were conducted in a thermostatically-controlled chamber (Figure 6.2). The incubation temperature was 25°C to improve the efficiency of contraction (Segal *et al.*, 1986). The incubation chamber was gassed with 95% O_2-5% CO_2, which also served to mix the medium. For each experiment, the proximal and distal tendons of the muscles were secured to the metal support and the force transducer respectively and then tensioned to give maximal force during stimulation (Figure 6.2).

After 10 min of preincubation, electrical stimulation was commenced using 200 ms trains of 100 Hz 0.5 ms pulses applied every 2 s and adjusted (10-12 V) to attain full fibre recruitment.

Tension development was recorded continuously during electrical stimulation. After the initial anaerobic phase, the contraction reached a sustained, aerobic, plateau level (15 min of stimulation). At this point either vehicle (control) or 1 μM 5-HT (including tracer amounts of [^{3}H]-5-HT and [^{14}C]-sucrose) was added to the incubation chamber, and contraction continued for a further 10 min. After cessation of stimulation, 5-HT-treated muscles were rapidly removed, weighed and freeze-dried. Freeze-dried muscles were homogenized in H_2O and after centrifugation an aliquot of supernatant was taken for radioactive counting to determine the total content of [^{3}H]-5-HT and [^{14}C]-sucrose. A further aliquot of supernatant was used for HPLC analysis (C18 reverse phase, mobile phase methanol:H_2O 90:10) to determine the metabolism of [^{3}H]-5-HT.

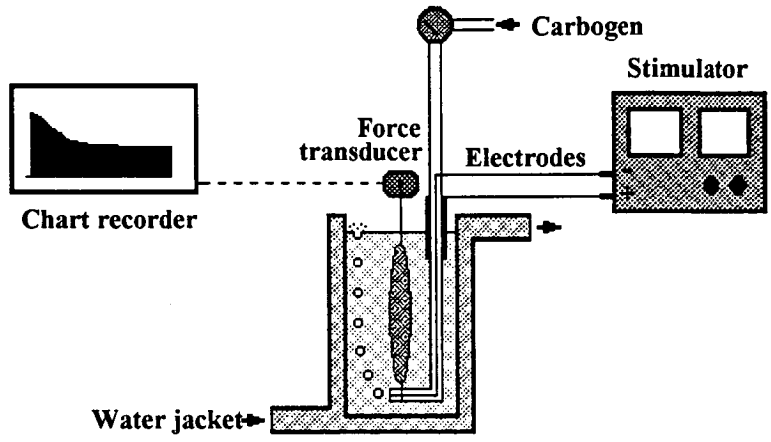

Figure 6.2 Isolated muscle incubation apparatus.

Soleus or EDL muscles were attached to the gassed, temperature-controlled incubation chamber and directly to the force transducer. 5-HT was added directly to the incubation medium. EDL, extensor digitorum longus; 5-HT, serotonin; carbogen, 95% O_2-5% CO_2.

6.2.3 Statistical analysis.

Two-tailed, unpaired Student's *t* test was used to determine the significance of difference between vehicle and 5-HT addition. Significant differences were recognized at $P < 0.05$.

6.3 Results.

Electrical stimulation of the sciatic nerve contracted all the muscle groups in the lower leg in addition to some muscle groups above the knee, consistent with the findings of Rennie and Holloszy (1977). Thus the observed changes in perfusion pressure and metabolism were a combination of contraction-stimulated lower leg, and uncontracted upper hindlimb effects.

6.3.1 Erythrocyte-free perfusions.

A distinct advantage of the perfused hindlimb at 25°C without erythrocytes was that venous P_{O_2} could be monitored continuously. Thus in the present experiments time course changes in $\dot{V}_{O_2}$ in relation to pressure and tension could be compared.

Under basal conditions, prior to electrical stimulation (flow rate 17.0 ± 0.2 $ml.min^{-1}$), mean arterial and venous P_{O_2} were 678.1 ± 8.4 mmHg and 593 ± 11.1 mmHg, respectively (n = 10) equating to a $\dot{V}_{O_2}$ of 8.0 ± 0.5 $\mu mol.h^{-1}.g^{-1}$. Basal perfusion pressure was 58.8 ± 1.2 mmHg (n = 10), and lactate release was 6.5 ± 1.2 $\mu mol.h^{-1}.g^{-1}$. The value for $\dot{V}_{O_2}$ under basal conditions was similar to that reported by this laboratory previously at flow rates greater than 8 $ml.min^{-1}$ (Ye *et al.*, 1990b).

Stimulation of the lower hindlimb muscle groups via the sciatic nerve caused an initial peak in tension development of 898 ± 50 g. The initial strong tension rapidly decreased to a plateau level of 211.1 ± 14.6 g after 6 min of stimulation (n = 10). The time taken to attain the plateau level of tension development coincided with the attainment of steady state for $\dot{V}_{O_2}$ and lactate release (approx. 4 min). After 6 min, $\dot{V}_{O_2}$ had increased to 12.3 ± 0.7* $\mu mol.h^{-1}.g^{-1}$ and lactate release to 24.0 ± 1.7*

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* Values for contraction-stimulated increases in $\dot{V}_{O_2}$ and lactate release were obtained using the entire hindlimb muscle mass, thus actual values/g contracting tissue may be 3 times greater [according to 30% contracting tissue, Rennie and Holloszy (1977)].

$\mu mol.h^{-1}.g^{-1}$. Perfusion pressure remained constant (59.5 ± 1.0 mmHg, n = 10) when vehicle alone was added. $\dot{V}O_2$ and tension development remained constant for the duration of the perfusion (Figure 6.4). Lactate release increased 4.4-fold with electrical stimulation and then decreased steadily during stimulation to a level approximately 2-fold greater than the basal pre-stimulation value.

6.3.1.1 Effect of prior infusion of 5-HT on skeletal muscle contraction.

Infusion of 0.25 μM 5-HT for 6 min increased perfusion pressure by 42.2 ± 7.5 mmHg and decreased $\dot{V}O_2$ and lactate release by 3.0 ± 0.4 $\mu mol.h^{-1}.g^{-1}$ and 3.9 ± 0.3 $\mu mol.h^{-1}.g^{-1}$, respectively (n = 3, Figure 6.3). Initial peak tension upon commencement of sciatic nerve stimulation was not affected by prior infusion of 5-HT (971 ± 26 g, n = 3, Figure 6.4). However, prior infusion of 5-HT inhibited plateau tension (6 min) development by 62.1 ± 16.3 %, and contraction stimulated rises in $\dot{V}O_2$ and lactate release by 75% and 40%, respectively (n = 3, Figure 6.3). During the stimulation period, 5-HT increased perfusion pressure a further 33.3 ± 13.8 mmHg (n = 3).

6.2.1.2 Effect of 5-HT during skeletal muscle contraction.

Figure 6.4 shows time courses for the effects of 0.25 μM 5-HT and its vehicle on tension development, $\dot{V}O_2$, lactate release and perfusion pressure. 5-HT inhibited plateau tension (6 min stimulation) development by 40%. The maximum inhibitory effect of 5-HT on tension development occurred 4 min after 5-HT infusion had ceased (Figure 6.4), and slowly returned to control levels by the end of the stimulation period. When 5-HT was infused for greater than two min, contraction did not return to control values even after 12 min of 5-HT removal (data not shown). Continuous recording of $\dot{V}O_2$ showed that the 5-HT mediated inhibition of $\dot{V}O_2$ preceded the inhibition of tension development (Figure 6.4). In addition, infusion of 5-HT almost (97%) totally inhibited the electrical stimulation-induced increase in $\dot{V}O_2$, with the inhibition occurring rapidly and coinciding with the increase in pressure (Figure 6.4). However, the inhibition reversed slowly and did not attain pre-5-HT infusion values until at least 5 min after the infusion of 5-HT had ceased.

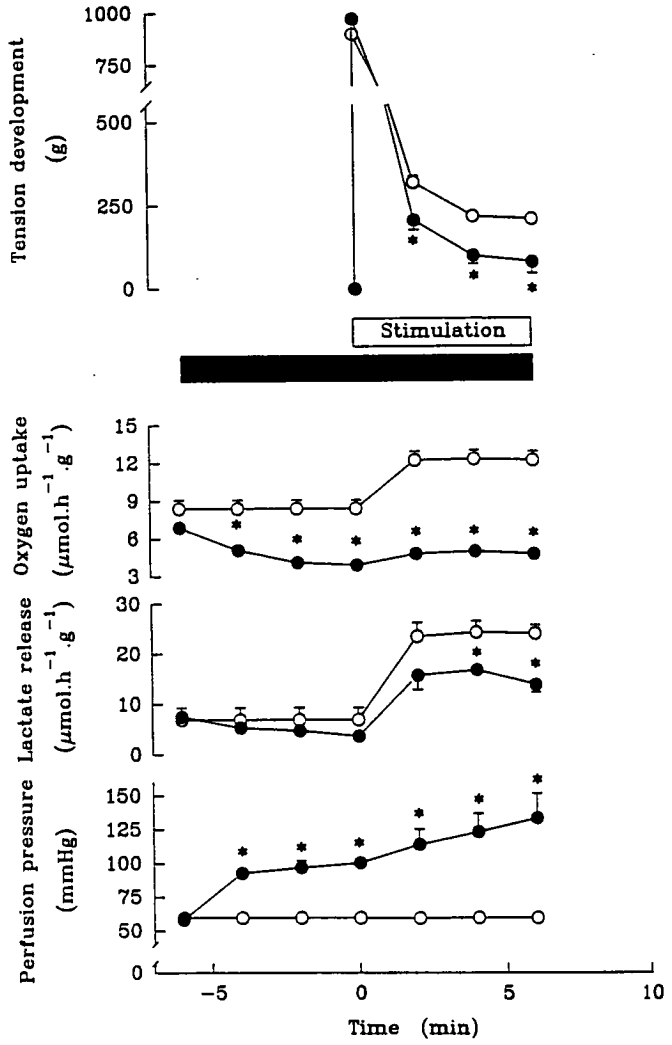

Figure 6.3 Effect of 5-HT on $\dot{V}O_2$, lactate release, perfusion pressure and tension development before and during skeletal muscle contraction.

The rat hindlimb was perfused in a non-recirculating mode without erythrocytes at 25°C as described in Methods. The lower hindlimb muscle groups were made to contract by electrical stimulation of the severed sciatic nerve (flank) using a suction electrode, and tension in the GPS muscle group was measured using an isometric force transducer. 5-HT (0.25 μM) or its vehicle were infused for the period indicated by the bar. Values are means ± SE for five control perfusions (O, from Figure 6.4) and three 5-HT-treated perfusions (●). When not visible, error bars are within symbols. 5-HT, serotonin, $\dot{V}O_2$, oxygen uptake; GPS, gastrocnemius-soleus-plantaris.

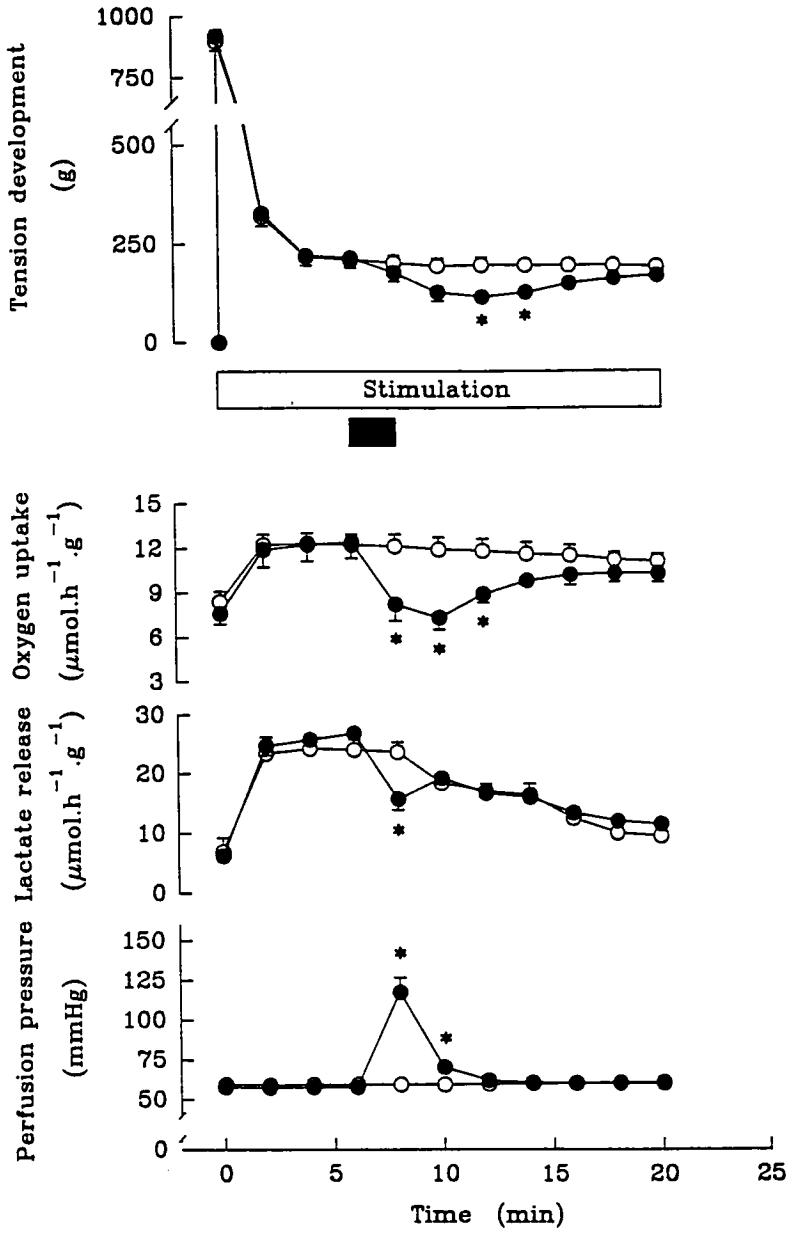

Figure 6.4 Effect of 5-HT on $\dot{V}O_2$, lactate release, perfusion pressure and tension development during skeletal muscle contraction.

The rat hindlimb was perfused in a non-recirculating mode without erythrocytes at 25°C and lower hindlimb muscles contracted as described in Figure 6.3. 5-HT (0.25 μM) or its vehicle were infused for the period indicated by the bar. Values are means ± SE for five control perfusions (O) and five 5-HT-treated perfusions (●). When not visible, error bars are within symbols. 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

Contraction-mediated lactate release was transiently inhibited by 41% after 2 min of 5-HT infusion, but within 2 min of removal of 5-HT, had returned to control values.

Infusion of 5-HT increased perfusion pressure by approx. 60 mmHg after 2 min of infusion, representing an increase of 103% above basal pressure. The time course for the change in pressure indicated that the overall effect lasted approx. 5 min, the pressure returning to control, untreated levels.

6.3.1 Erythrocyte perfusions.

6.3.1.1 Effect of 5-HT during skeletal muscle contraction.

Under basal conditions at high flow rate (15.3 ± 0.2 $ml.min^{-1}$), mean arterial oxygen content was 7.89 ± 0.12 mmol/l, mean venous oxygen content was 6.88 ± 0.13 mmol/l (n = 10) with a $\dot{V}O_2$ of 59.6 ± 3.1 $\mu mol.h^{-1}.g^{-1}$, and lactate release was 12.3 ± 2.1 $\mu mol.h^{-1}.g^{-1}$. The values for $\dot{V}O_2$ and lactate release under basal conditions were somewhat higher than those reported by Ruderman *et al.* (1971) but may result from the higher flow rate used in the present studies and test stimulations of the muscles to generate the force tension curve. $\dot{V}O_2$ (Ruderman et al., 1980; Ye *et al.*, 1990b) and lactate release (Ye *et al.*, 1990b) are flow rate dependent in the perfused rat hindlimb. Basal perfusion pressure was 64 ± 2 mmHg (n = 8, Figure 6.5), which was higher than erythrocyte-free perfusions despite similar flow rates. This was presumably due to the higher viscosity of the 4% BSA erythrocyte medium.

Figure 6.5 shows the effect of electrical stimulation of the sciatic nerve on tension development, lactate release and perfusion pressure. Stimulation of the lower hindlimb muscle groups via the sciatic nerve produced an initial tension in the Achilles tendon of 1109 ± 127 g (n = 5) and 1082 ± 95 g (n = 5) for the control and 5-HT treated groups, respectively. These values were similar to that initially developed by the erythrocyte-free perfused hindlimb at 25°C. Tension then rapidly decreased to a plateau level of 335 ± 28 g (n = 5), control group and 312 ± 40 g (n = 5), 5-HT treated group, which was approx. 150% of that attained using erythrocyte-free perfused hindlimbs at 25°C, and remained constant for the duration of the perfusion (Figure 6.5). Lactate release initially increased 5-fold with electrical stimulation then decreased slightly to 2-fold basal levels (Figure 6.5). The perfusion pressure rose gradually by approx. 8 mmHg in the course of the perfusions. Stimulation *per se*

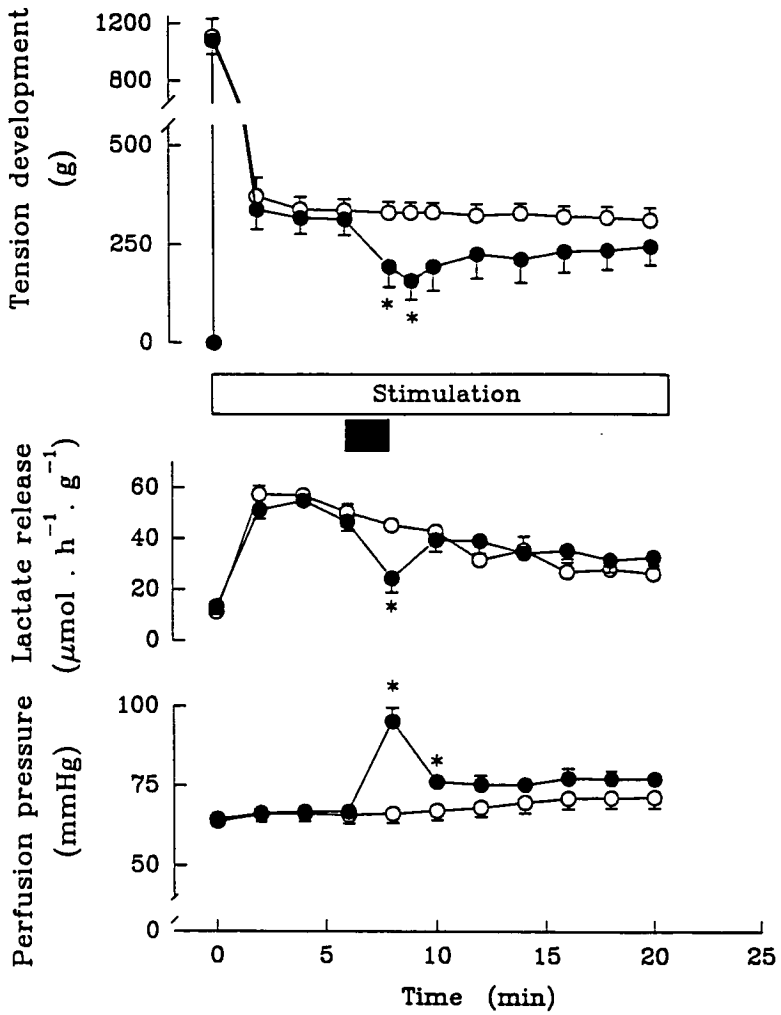

Figure 6.5 Effect of 5-HT on lactate release, perfusion pressure and tension development during skeletal muscle contraction.

The rat hindlimb was perfused in a non-recirculating mode with medium containing erythrocytes at 37°C, and lower hindlimb muscles contracted as described in Figure 6.3. 5-HT (0.25 μM) or its vehicle were infused for the period indicated by the bar. Values are means ± SE for five control perfusions (O) and five 5-HT-treated perfusions (●). When not visible, error bars are within symbols. 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake.

appeared to have no immediate effect on perfusion pressure (Figure 6.5). The initial effect of electrical stimulation on $\dot{V}O_2$ could not be quantitatively assessed as timing of sampling to correspond precisely with peak tension development was not possible. However continuous recording of the venous PO_2 in some of the erythrocyte-perfusions suggested that there was no immediate change during this period. Table 6.1 shows that $\dot{V}O_2$ increased 1.5-fold during stimulation from 61.1 ± 4.1 to 92.5 ± 5.9 $\mu mol.h^{-1}.g^{-1}$. Again, continuous recordings suggested that this took 2-3 min to reach steady state (data not shown). Contraction-stimulated rises in $\dot{V}O_2$ in erythrocyte-perfused hindlimbs when expressed per gram of contracting muscle are comparable to values obtained by others (Table 6.2).

Figure 6.5 shows that a brief (2 min) infusion of 0.25 μM 5-HT, commenced 6 min after the start of electrical stimulation, increased perfusion pressure by approx. 30 mmHg. Continuous recording of data showed that the effect was evident within seconds after commencement of infusion and began to rapidly reverse as soon as infusion ceased. However a residual pressure increase was consistently present for the remainder of the stimulation period. This is in contrast to the complete reversal of pressure effects in the absence of erythrocytes (Figure 6.4). When 5-HT was infused for longer than 2 min, the reversal of pressure after 5-HT removal was slower and remained elevated above control (data not shown). The increase in pressure was less than the change in pressure in the erythrocyte-free perfused hindlimb at 25°C (Figure 6.4).

Figure 6.5 also shows that after 3 min following 5-HT removal, there was a 54% decrease in developed tension during the plateau aerobic phase from 329.3 ± 25.2 g (n = 5) to 156.5 ± 47.9 g (n = 5). Tension slowly returned towards pre-5-HT levels, but remained below control values for the remainder of the stimulation period (Figure 6.4).

Contraction-mediated lactate release in was transiently inhibited by 39% during 5-HT infusion from corresponding control values of 45.1 ± 2.3 $\mu mol.h^{-1}.g^{-1}$ to 24.4 ± 5.6 $\mu mol.h^{-1}.g^{-1}$ after 2 min of 5-HT infusion. Within a further 2 min lactate release had returned to control values, which continually declined for the remainder of the stimulation period (Figure 6.5).

Perfusion Condition	Time (min)	$\dot{V}O_2$ ($\mu mol.h^{-1}.g^{-1}$) Vehicle	$\dot{V}O_2$ ($\mu mol.h^{-1}.g^{-1}$) 0.25 μM 5-HT
Basal	0	61.1 ± 4.1	58.1 ± 5.1
Plateau Contraction*	6	92.5 ± 5.9	98.4 ± 12.4
Maximal Response to 5-HT*	9	95.1 ± 9.6	64.6 ± 9.5
End Contraction Period	19	71.8 ± 6.9	73.1 ± 15.1

Table 6.1 Effect of 5-HT on $\dot{V}O_2$ by perfused hindlimb during skeletal muscle contraction.

The rat hindlimb was perfused and lower hindlimb muscles contracted as described in Figure 6.5. Venous effluent was sampled prior to and during contraction stimulation at the times indicated, and analysed as described in Methods. 5-HT (0.25 μM) or its vehicle were infused for a two min period after plateau tension was obtained (Figure 6.5). Values are means ± SE for five control perfusions and five 5-HT-treated perfusions. 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake; *, samples collected at times corresponding to changes in venous PO_2 observed with an in-line oxygen electrode.

Ref.	Hindlimb Mass Contracted	$\Delta \dot{V}O_2$ ($\mu mol.h^{-1}.g^{-1}$)	
		Basal*	Contraction§
This Study	30%[1.]	59.6 ± 3.1	119.3
Ruderman *et al.* (1971)	30%[1.]	14.6 ± 1.6	142.0
Ruderman *et al.* (1971)†	30%[1.]	18.2 ± 2.4	90.7
Richter *et al.* (1982a)†	30%[1.]	23.5 ± 1.1	128.3
Spriet *et al.* (1985)	56%[A]	33.6 ± 1.3	122.0
M[C]Allister and Terjung (1991)†	80%[A]	20.4 ± 1.2	194.3

Table 6.2 Basal and contraction-stimulated $\dot{V}O_2$ in erythrocyte-perfused rat hindlimbs.

Hindlimbs or lower-limbs (#) of sedentary rats were perfused with constant, high flow rates at 37°C with modified Krebs-Henseleit medium containing either fresh or rejuvenated erythrocytes and 4% bovine serum albumin. Portions (% given) of the perfused mass were electrically stimulated to contract via the sciatic nerve. Arterial and venous effluent samples were analysed with blood-gas analysers. *, Values are means ± SE; §, maximum contraction-stimulated increases in $\dot{V}O_2$ were recalculated assuming that only contracting tissue contributed to the rises in $\dot{V}O_2$; A, from Rennie and Holloszy (1977); †, medium contained insulin.

Contraction-induced $\dot{V}O_2$ was markedly inhibited (94%) by 5-HT (Table 6.1), but after 13 min following 5-HT removal, $\dot{V}O_2$ had returned to control levels (Table 6.1).

6.3.3 Incubated muscles.

Comparison of all the data from both control and 5-HT groups showed that before addition of vehicle or 5-HT, electrical stimulation of the soleus and EDL muscles produced significantly different ($P < 0.01$) initial tensions of 22.8 ± 1.1 g and 35.3 ± 2.7 g respectively ($n = 6$). After 15 min of stimulation, the tension rapidly decreased to significantly different (soleus *vs.* EDL) plateau levels of 11.4 ± 0.6 g and 7.3 ± 0.5 g respectively ($P < 0.001$, $n = 6$) and remained relatively constant for the remainder of the incubation (Figure 6.6).

Figure 6.6 shows that 5-HT had no significant effect on the tension developed by either the soleus or EDL muscles. This lack of an effect was not the result of poor penetration or excessive metabolism of 5-HT as [^{3}H]-5-HT diffused into, but was not significantly metabolized by the muscles (data not shown).

6.4 Discussion.

The main observation from this study was that 5-HT inhibited the aerobic contractility of working muscle in the constant-flow perfused rat hindlimb, but not incubated muscles. This effect appeared to have occurred as a result of impaired nutrient delivery and was evident whether or not 5-HT was infused before and/or during the stimulation period.

Two perfusion systems were used in the present study. The perfusions with erythrocytes at 37°C were conducted because of the possibility that oxygen delivery to working muscle would be inadequate if erythrocytes were omitted. However a disadvantage of the erythrocyte-perfused system was that continuous recording of $\dot{V}O_2$ was not readily possible and intermittent perfusate samples were taken for oxygen content analysis. Perfusion with erythrocyte-free medium overcame this problem.

The present studies show that the effects of both sciatic nerve stimulation and 5-HT were qualitatively similar for both types of perfusion. As shown in Figures 6.4 and 6.5, during contraction, tension produced by the lower hindlimb muscle

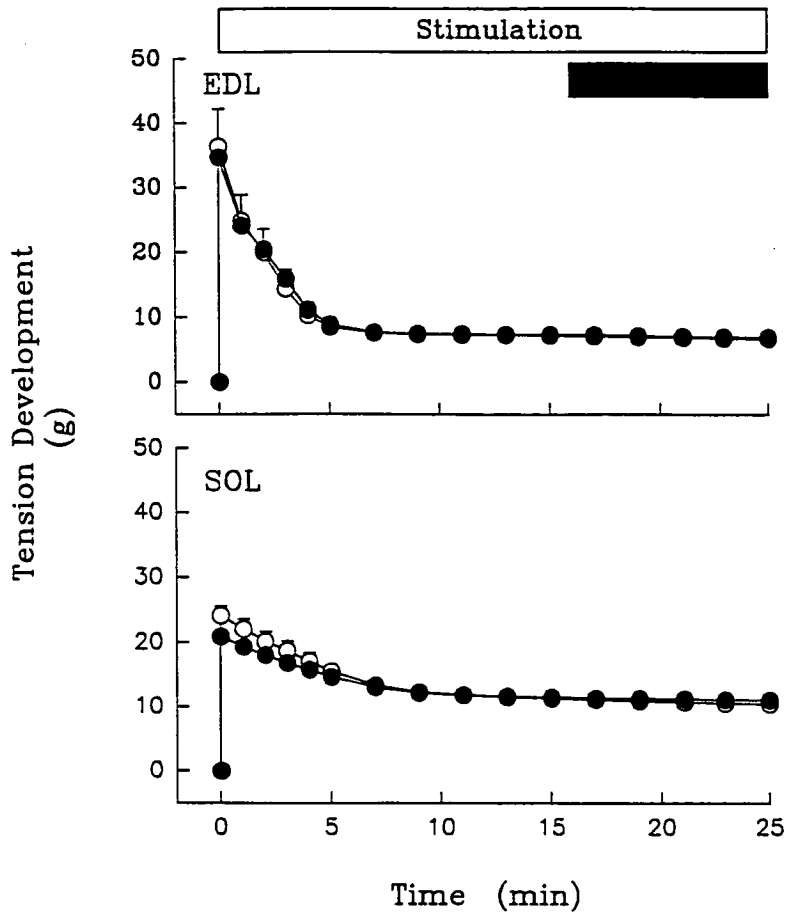

Figure 6.6 Effect of 5-HT on muscle tension development by electrically-stimulated isolated soleus and EDL muscles.

Muscles were dissected, incubated and stimulated as described in Methods. Either the EDL or soleus muscles were attached to the incubation apparatus and electrically stimulated to contract. Tension generated by each muscle was measured using an isometric force transducer. Values are means ± SE with 3 incubations for each treatment. Vehicle (O) or 5-HT (●, 1 μM) were added 15 min after commencement of electrical stimulation and were present for the period indicated by the filled bar. 5-HT, serotonin; $\dot{V}O_2$, oxygen uptake; EDL, extensor digitorum longus; SOL, soleus.

groups fatigued after an initial peak in tension. Richter (1984) proposed that the rapid initial fall in tension could be due to selective fatigue of the fast-twitch white fibres resulting from rapid glycogen depletion (Richter *et al.*, 1982b). These fibres constitute approximately 60% of the gastrocnemius-plantaris-soleus group (Ariano *et al.*, 1973). Consistent with this report, the incubated EDL had a significantly greater initial tension when compared to the soleus, but fatigued to plateau levels significantly below the soleus (Figure 6.6).

In both perfusion systems, sciatic nerve stimulation led to marked increases in $\dot{V}O_2$ and lactate release in association with tension development but little change in perfusion pressure. 5-HT increased perfusion pressure, markedly decreased tension, and contraction-induced $\dot{V}O_2$ and, to a lesser extent, lactate release in both perfusion systems. The latter suggests anaerobic metabolism was the predominant generator of energy during 5-HT-impaired contraction. The rapid reversal of the lactate release to control levels may also be due, at least in part, to the transient increases in lactate release observed after 5-HT removal (Dora *et al.*, 1991, Chapter 2). This washout was presumably due to re-establishment of flow to previously oxygen-limited regions, and was similar to that observed upon re-establishment of flow to physically occluded regions (Chapter 2, Dora *et al.*, 1991).

However there were differences between the two perfusion systems that merit comment. Whilst peak tension development of the muscle group was similar in the two preparations, plateau tension development differed considerably with 324 ± 23 and 211 ± 15 g, being the tension developed with erythrocyte perfusion at 37°C, and erythrocyte-free perfusion at 25°C, respectively[1]. This difference may result

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1. Although not directly relevant to the present study on the effects of 5-HT, a comparison of Figures 7.4 and 7.5 reveals that the perfused hindlimb appears to perform more efficiently at 25°C without erythrocytes than at 37°C with erythrocytes. At 25°C the hindlimb developed 211 ± 15 g of tension and consumed an extra 3.8 μmol $O_2.h^{-1}.g^{-1}$, equivalent to 55.5 g of tension/μmol $O_2.h^{-1}.g^{-1}$. At 37°C the ratio was far lower at 9.1 g of tension/μmol $O_2.h^{-1}.g^{-1}$. Contraction-stimulated lactate release at 25°C was half that at 37°C. Thus at 25°C and 37°C the ratios of tension:lactate release were 11.2 and 9.0 g of tension/μmol lactate.$h^{-1}.g^{-1}$, respectively. There is no obvious explanation why tension development is more expensive at 37°C with erythrocyte-perfused hindlimbs.

from the superior oxygen delivery afforded by erythrocytes, or it may reflect lower efficiency of excitation-contraction coupling, and/or muscle performance, at the lower temperature (Stephenson & Williams, 1981).

Another major difference between the two perfusion systems was the magnitude of the effect of 5-HT. When infused within the contraction period, during erythrocyte perfusions at 37°C, 0.25 μM 5-HT resulted in a pressure increase of approximately 30 mmHg. This compared to an increase of 60 mmHg in the erythrocyte-free perfusions at 25°C. An explanation as to why 5-HT-mediated vasoconstriction was less pronounced at 37°C may relate to a right-ward shift in the dose response curve for 5-HT at the higher temperature (Chapter 5, Figure 5.1), consistent with the observed increase in 5-HT pressure-sensitivity with cooling (Harker *et al.*, 1991). However, when infused in the absence of contraction at 25°C, 0.25 μM 5-HT increased pressure to steady-state levels only 42 mmHg above basal.

Time course studies using erythrocyte-free perfused hindlimbs at 25°C (Figure 6.4) showed that the sequence of changes induced by 5-HT was increased perfusion pressure (vasoconstriction) closely associated with an inhibition of $\dot{V}O_2$, followed by decreased contractility. This sequence and the lack of an effect in incubated muscles suggests that 5-HT does not impair contractility directly by receptor-mediated effects on skeletal muscle. Rather, the sequence is consistent with the hypothesis of functional vascular shunting (Dora *et al.*, 1991, 1992). So far the evidence supporting the notion of 5-HT mediated vascular shunting in perfused rat hindlimb is indirect (Chapters 2, 3, and 5). In the present study it is proposed that 5-HT constricted larger blood vessels, possibly feed arteries, to induce a partial redistribution of flow away from the working muscles thereby diminishing oxygen supply. This effect may have been fibre-type specific, presumably with flow decreasing to slow-oxidative fibres which have less capacity for anaerobic contraction metabolism. The feed arteries have been shown to be active sites of blood flow control to contracting muscle, with soleus muscles exhibiting a greater range of flow control than EDL (Williams & Segal, 1993).

The effect of 5-HT to redistribute perfusate flow and concomitantly decrease contractility in the perfused hindlimb are similar to effects of sympathetic nervous system activity observed *in vivo*. During low intensity exercise *in vivo*, in addition to local vasodilatation, there is also an increase in sympathetic nervous system activity, predominantly to redistribute cardiac output away from inactive areas. However, as

exercise intensity increases, the maximal flow to contracting muscle can be limited by sympathetic vasoconstriction (Joyner *et al.*, 1990, 1992). This occurs at the level of the feed arteries external to the active muscle (Lind & Williams, 1979), overcoming the contraction-induced dilatation suggesting diminished effectiveness of upstream communication (as reviewed by Segal, 1992).

It is unknown whether upstream communication occurs in the constant-flow, perfused rat hindlimb preparation used in these studies. However, using the same perfusion system, exercise-stimulated vasodilator release decreased the vasoconstriction by angiotensin II (Colquhoun *et al.*, 1990). Skeletal muscle contraction did not overcome the 5-HT-induced vasoconstriction in this study thus providing evidence supporting the notion of 5-HT stimulating vasoconstriction at different sites to thermogenic vasoconstrictors (such as angiotensin II) within the muscle bed.

CHAPTER 7

Vasoconstrictor-mediated alterations in flow distribution.

7.1 Introduction.

The results in this thesis have clearly demonstrated that vasoconstriction in the constant-flow perfused rat hindlimb can differentially control tissue metabolism. It has been proposed that vasoconstriction by low doses of norepinephrine (NE) leading to increases in hindlimb oxygen uptake ($\dot{V}O_2$) and lactate release was at least partly due to work done during constriction of arteriolar smooth muscle, and a possible redistribution or recruitment of flow to highly metabolically active regions (described in more detail in Chapter 1). Conversely, vasoconstriction by serotonin (5-HT) and high doses of NE leading to decreases in hindlimb $\dot{V}O_2$ and lactate release appeared to occur at sites metabolically distinct from lower doses of NE. Since 5-HT is known to constrict relatively larger blood vessels than those constricted by angiotensin II, vasopressin and low doses of NE (Gray, 1971; Blackshear *et al.*, 1985; Hirst & Edwards, 1989; Lamping *et al.*, 1989; Tuncer *et al.*, 1992), partial or complete closure of the 5-HT-sensitive vessels may either redistribute flow to less metabolically active tissue, or deny flow to some regions.

These apparently anomalous metabolic effects of vasoconstrictors do not appear to be due to direct effects on skeletal muscle metabolism and therefore raise questions about the control of flow through the rat hindlimb vasculature. Techniques used to measure changes in flow within the microvasculature of an organ include:

(a) dilution measurements of bolus injected nondiffusible indicators such as dyes (Starr & Frasher, 1975) and labelled erythrocytes or dextrans (Baker *et al.*, 1982); or diffusible indicators such as radiolabelled xenon (Lassen, 1964) or other gasses (Grønlund *et al.*, 1989; Harrison *et al.*, 1990b);

(b) entrapment of bolus injected radio- or fluorescently-labelled markers such as microspheres (Laughlin *et al.*, 1982; Piiper *et al.*, 1985; Gorski *et al.*, 1986; Iversen & Nicolaysen, 1990; Bassingthwaighte *et al.*, 1987, 1990), or soluble flow markers (Bassingthwaighte *et al.*, 1990);

(c) measurement of erythrocyte velocities and flow distribution in transilluminated surface capillaries of muscles using intravascular microscopy

(Eriksson & Myrhage, 1972; Sullivan & Pittman, 1982; Lindbom & Arfors, 1984; DeLano *et al.*, 1991; Tyml & Budreau, 1991); and

(d) physical observation of vascular architecture by digestion or clearing tissue surrounding methyl-methacrylate (Potter & Groom, 1983; Kimura *et al.*, 1990) or silicone rubber (Williams & Segal, 1992) casts.

Each of the above techniques has inherent advantages and disadvantages (reviewed by Hyman, 1971; Bassingthwaighte, 1986; Duling & Damon, 1987; Austin *et al.*, 1989; Lipowsky *et al.*, 1988). Indicator dilution techniques can be carried out on intact, undisturbed tissues, and most do not alter flow patterns. However, vascular tissue adherence and permeability can be limitations to accuracy of measurement. Microspheres do not leave the lumen, yet their use has many disadvantages. These include: partial or complete obstruction of flow through vessels in which they lodge; alteration of the apparent size of vessels when forced through by backpressure; no indication of flow heterogeneity within capillaries; the occurrence of rheological biasing; and, when using radiolabelled spheres, stochastic and reference errors must be considered (reviewed by Austin *et al.*, 1989). Both flow markers and microspheres have the potential to show temporal alterations in the distribution of flow, but do not distinguish true flow heterogeneity from spatial heterogeneity (Duling and Damon, 1987) and do not give an indication of relative tissue metabolism. Vascular corrosion casts usually contain toxic components and have high viscosity. These factors can alter flow patterns within the circulation (reviewed by Lametschwandtner *et al.*, 1990), especially in the presence of vasoconstrictors.

In this study, the techniques employed were dye efflux kinetics, entrapment of labelled dextran and injection of microspheres.

7.2 Materials and methods.

7.2.1 Perfused hindlimbs.

Surgery was performed on 180-200 g rats as outlined in Section 2.2.1.2.1. Experiments were performed at 25°C with 2% BSA added to the perfusion medium. Additional details are given in Section 2.2.1. In fluorescein-labelled dextran entrapment studies both legs of each rat were perfused. This minimized the possibility of pressure-induced redistribution of flow through collateral circulation. In these experiments the perfusion protocol was as previously except the contralateral

common iliac was not tied and the perfusion flow rate was doubled. The hindlimb skeletal muscle mass was assumed to be twice that of one hindlimb.

7.2.2 Evans Blue dye efflux kinetics.

A dye injection technique was used to estimate changes in volume of tissue perfused. Evans Blue dye was used as it could be detected during the experimental period by use of an in-line spectrophotometer. A constant volume injector (Pharmacia Fine Chemicals, Sweden) was incorporated into the hindlimb perfusion apparatus prior to the hindlimb. This enabled a 35 μl pulse of filtered (Millipore 0.45 μm) Evans Blue dye (5 $mg.min^{-1}$ oxygenated perfusion media) to be injected into the arterial line with minimal, and transient alteration in hindlimb pressure. A Hitachi model 101 spectrophotometer was positioned immediately beyond the hindlimb, and by incorporating an in-line flow-through cuvette, continuous changes in absorbance (630 nm) of venous perfusate were monitored. The initial increase in absorbance was rapid relative to decrease from maximum, thus data were only analysed for the latter. In addition, due to technical limitations, mean transit times through the hindlimb could not be monitored, thereby not permitting the Stewart-Hamilton technique of indicator dilution (described by Lipowsky *et al.*, 1988).

Dye absorbance was linearly related to dye concentration. At steady state $\dot{V}O_2$ and pressure and when the effluent was free of residual erythrocytes, dye injections were commenced. Absorbance was recorded continuously and three separate injections of dye were made once steady state conditions were obtained before, during, and after infusion of 5-HT or NE. To assess the effect of a deliberate diminution of perfused tissue the hindlimb was reversibly tied off below the knee with a soft rubber tourniquet, thereby decreasing the perfused muscle mass from approximate values of 15 to 12.5 g, and dye was injected as above. A separate rat was used for each treatment.

Reproducibility and rapidity of efflux profiles suggested leakage from the lumen was minimal, or at least consistent. When studying flow through the feline mesentery, Starr and Frasher (1975) reported than in the presence of 3% bovine albumin, a solution of 5% Evans blue did not produce vascular leakage or any other adverse effect visible through a microscope.

7.2.3 Entrapment of fluorescein-labelled dextran.

Fluorescein-labelled dextran (Fluorescein isothiocyanate, FITC, av. mol. wt. 150,000, Pharmacia Fine Chemicals, USA) was used in this study to reduce the possibility of vascular leakage and to enable detection at low concentrations without the need for radioisotopes. Svensjö *et al.* (1978) found that upon intravenous injection of FITC-dextran (av. mol. wt. 150,000) only a few leakage sites per cm^2 of cheek pouch or cremaster muscle were observed, mostly through postcapillary venules. However, the number of leakage sites increased upon topical application of histamine or bradykinin (Svensjö *et al.* (1978).

5-HT has also been shown to produce increases in vascular permeability to macromolecules subsequent to endothelial cell separation in the postcapillary venules (reviewed by Grega, 1986). Thus in preliminary studies, the washout of tracer amounts of [^{14}C]-sucrose ($2x10^4$ $dpm.ml^{-1}$ in 0.5 mM unlabelled sucrose) in addition to FITC-dextran was determined. In situations where the sucrose space was increased due to tissue oedema, the sucrose washout was delayed, but FITC-dextran washout was not markedly affected (data not shown). In addition, FITC-dextran washout patterns were compared in the presence and absence of 5-HT, and no significant difference observed (data not shown).

FITC-dextran was dissolved in perfusion medium to a final concentration of 1 $mg.ml^{-1}$ and infused into the hindlimb at one-hundredth the perfusion flow rate. Fluorescence was detected using an Aminco-Bowman Spectrofluorometer, excitation and emission wavelengths set at 494 and 528 nm, respectively. Venous effluent samples (0.5, 1 or 2 min) were collected with an ISCO Retriever II fraction collector and centrifuged to remove erythrocytes (IEC DPR-6000 centrifuge, 2000 g). After 10 min of FITC-dextran infusion, and in the presence of FITC-dextran, either NE (0.1 μM) or 5-HT (1 μM) were infused for a minimum of 10 min until steady state pressure and $\dot{V}O_2$ were obtained. The protocol then involved either:

Protocol *I*: Removal of the agonist until basal $\dot{V}O_2$ and perfusion pressure were obtained (15 min) then cessation of FITC-dextran infusion. After a defined period (t = 6, 10 or 15 min) the agonist was reinfused and measurement of washout of entrapped marker commenced (Figure 7.2); or

Protocol *II*: Cessation of FITC-dextran infusion after a defined period (t = 6, 10 or 15 min) before removal of the agonist and measurement of washout of entrapped marker (Figure 7.2).

The difference in the two protocols was Protocol *I* involved agonist-induced entrapment in a recruited space, whereas Protocol *II* involved agonist-induced entrapment in a space denied access from the basal-perfused space. t was varied to determine whether entrapped spaces were slowly perfused or totally denied flow.

The summed differences between control and treated washout curves were transformed to FITC-dextran concentrations from standard curves. The vascular volumes could be estimated by comparison to the steady-state concentration of FITC-dextran during infusion.

The total basal vascular space was also determined to allow determination of the proportion entrapped by agonists. The FITC-dextran was infused and allowed to equilibrate in the untreated hindlimb. The rat was disconnected, marker removed from the apparatus inflow and outflow tubing, and the rat reconnected (4 min unperfused). The washout from the hindlimb was then determined. The volume contained in the arterial and venous cannulas was also determined and subtracted from space values.

7.2.4 Microsphere injection.

To assess whether 5-HT-induced vasoconstriction was redistributing flow through direct arterio-venous (A-V) shunts in the hindlimb, microspheres were injected in the absence and presence of 1 μM 5-HT.

The size distribution of the polystyrene spheres (Latex Beads, aqueous suspension, Sigma, USA) was determined to be 12.12 ± 2.93 μm (mean ± SD, n = 177), ranging in size from 5-20 μm (Figure 7.2). 15 μl (1-3 x 10^6) of the microsphere suspension was added to 1.5 ml perfusion medium and sonicated for 1 hr to break up aggregates.

In control perfusions, the sonicated suspension was drawn into a 1 ml syringe and rapidly injected through a 3-way tap into the arterial perfusion line. The other arm of the tap was used for flushing.

In 5-HT-treated hindlimbs, the sonicated suspension was drawn into a low dead-space 1 ml syringe with needle built in (27G, Terumo, USA) and injected into

the arterial line through the infusion port.

Immediately upon injection of the microspheres, effluent perfusate was collected directly into centrifuge tubes in 5 min fractions for 10 min. Samples were centrifuged for 15 min at 2000 g, and the supernatant aspirated. The pellet was resuspended and transferred after weighing to an eppendorf tube. 10 µl of detergent (DECON 90, Selby Anax, Australia) was added to dissolve erythrocyte membranes. All samples were vortex mixed and sonicated for 1 hr before counting using a light microscope and haemocytometer.

7.2.5 Statistical analysis.

The statistical significance of differences between groups of data were assessed by either paired or unpaired, two-tailed Student's *t* test. Significant differences were recognized at $P < 0.05$.

The exponential equations in Figure 7.4 were iteratively fitted using EXPFIT (Guardabasso *et al.*, 1991).

7.3 Results.

7.3.1 Evans Blue dye efflux kinetics.

The steady state mean perfusate PO_2 values for the rat hindlimb were 687 ± 8 mmHg arterial and 443 ± 8 mmHg venous ($n = 11$). These values were obtained after a 30 min equilibration period. The basal $\dot{V}O_2$ was found to be 5.9 ± 0.2 µmol.h^{-1}.g^{-1} ($n = 11$). Basal steady state lactate production was 8.4 ± 0.3 µmol.h^{-1}.g^{-1} ($n = 8$) and basal pressure was 24 ± 1 mmHg ($n = 11$). The basal steady state values for $\dot{V}O_2$, perfusion pressure and lactate release remained constant for the duration of control experiments (vehicle only) and were similar to those reported previously from this laboratory (Colquhoun *et al.*, 1988, 1990; Hettiarachchi *et al.*, 1992).

Figure 7.1 shows the results of the dye injection studies. Three injections of Evans Blue dye were made before, during and after infusion of agonists or occlusion of the hindlimb. Averages for each set of three injections were calculated and thereafter pooled between rats to obtain means ± SE for the control perfusion and each treatment. No significant difference was noted between the averages before and after each treatment. Values for absorbance were plotted as a function of time with

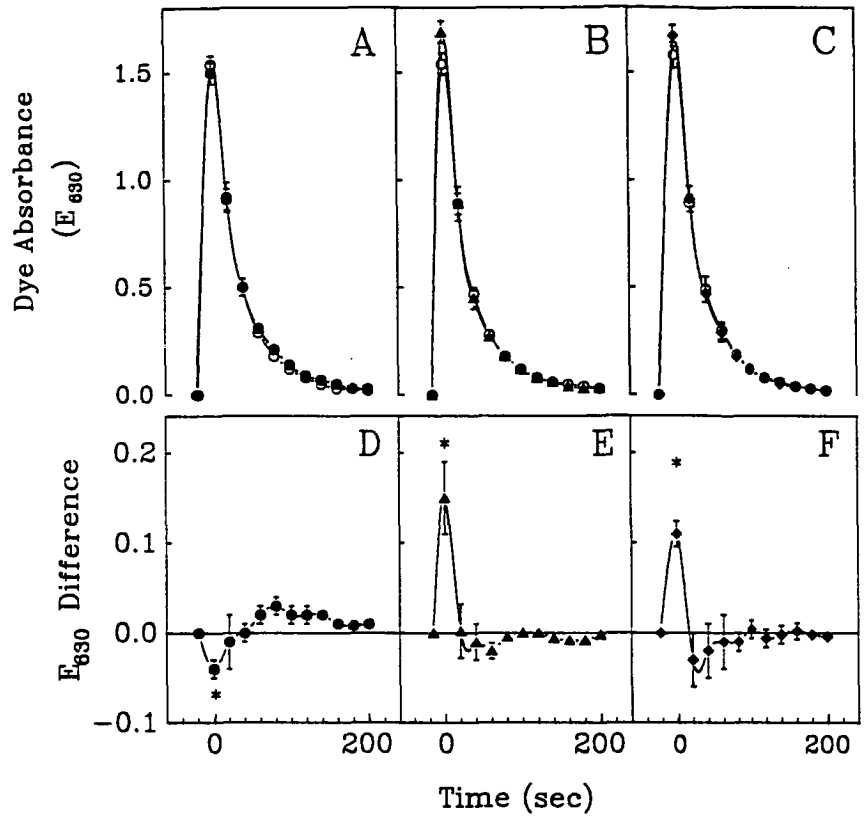

Figure 7.1 Dye washout profiles and difference curves in the constant-flow perfused rat hindlimb.

Hindlimbs from 180-200 g rats were perfused at 25°C and a constant flow rate of 4 ml.min^{-1} with perfusion medium containing 2% bovine serum albumin and gassed with 95% O_2-5% CO_2. Panel **A** shows the effect of infusion of NE (●, 75 nM) on the time course for Evans Blue dye washout. Panel **B** shows the effect of 5-HT (▲, 0.5 μM) and panel **C** shows the effect of reversible hindlimb occlusion at the knee (◆). Panels **A-C** also show data for the control conditions involving no infusion of agonist or muscle occlusion (O). Panels **D**,**E**, and **F** show the respective difference curves obtained by subtraction of control (no addition) values. Dye was injected under steady state conditions, further experimental details are given in Methods. Averages for each set of three injections within each experiment were calculated. Values shown are means ± SE of the averages from five different rats for each separate treatment. *, significantly different from corresponding control value ($P < 0.05$), paired Student's *t* test. NE, norepinephrine; 5-HT, serotonin.

From Dora *et al.* (1991).

the maximum value arbitrarily at 0 time as this maximum value of effluent dye absorbance represents the most common path length or transit time through the perfused tissue. Assuming that passage of dye through functional shunts is quicker because of shorter length and faster flow than through capillary beds, the peak value is inversely related to the volume of capillary bed or volume of tissue perfused. The results indicate that NE at a dose which increased pressure by 31 ± 5 mmHg and $\dot{V}O_2$ by 4.4 ± 0.4 μmol.h^{-1}.g^{-1} (68%, n = 5) significantly decreased maximum absorbance values from control values of 1.54 ± 0.04 to 1.50 ± 0.05 (3%). Conversely at a dose which increased pressure to the same extent as that observed with NE (31 ± 2 mmHg), yet decreased $\dot{V}O_2$ by 1.8 ± 0.3 μmol.h^{-1}.g^{-1} (30%, n = 5), 5-HT significantly increased maximum absorbance from 1.54 ± 0.05 to 1.69 ± 0.05 (9%). The occluded hindlimb decreased $\dot{V}O_2$ by 1.3 ± 0.2 μmol.h^{-1}.g^{-1} (23%) yet increased maximum absorbance from 1.57 ± 0.06 to 1.67 ± 0.05 (6%). Since dye concentration and total flow remain constant, these observed changes in maximum absorbance are reflected in changes in rate of dye washout; 5-HT and the occluded hindlimb inducing a more rapid washout than observed with control conditions and NE a lesser decrease in rate of dye washout. Upon infusion of NE the presence of a small quantity of erythrocytes was observed in the perfusate which were not observed upon infusion of 5-HT. Doses of NE greater than 0.1 μM, which involved increases in pressure greater than 40 mmHg yet had reached maximal increases in $\dot{V}O_2$, led to an alteration in the dye washout curve such that maximum absorbance values were further decreased.

7.3.2 Entrapment of fluorescein-labelled dextran.

In experiments where two hindlimbs were perfused, the steady state mean perfusate PO_2 values for the rat hindlimb were 692 ± 7 mmHg arterial and 402 ± 19 mmHg venous (n = 27). These values were obtained after a 30 min equilibration period. The basal $\dot{V}O_2$ (assuming the perfused skeletal muscle mass was twice that of one perfused hindlimb) was found to be 6.9 ± 0.2 μmol.h^{-1}.g^{-1}, and basal perfusion pressure was 23 ± 1 mmHg (n = 27), neither value significantly different to the basal values obtained in Chapter 2 for one hindlimb. Basal lactate release, however, was significantly lower ($P < 0.01$) at 4.1 ± 0.4 (n = 5). This value is within the range of values obtained for one hindlimb.

During the entrapment experiments 0.1 μM NE increased $\dot{V}O_2$ by 3.1 ± 0.1

$\mu mol.h^{-1}.g^{-1}$ (43 ± 4 %) and perfusion pressure by 32 ± 2 mmHg (142 ± 22 %) above basal values. Both these proportions were significantly ($P < 0.001$, $P < 0.02$, respectively) less than the corresponding effects of NE when one hindlimb was perfused. However in a separate study, 0.25 μM NE increased $\dot{V}O_2$ by 3.9 ± 0.5 $\mu mol.h^{-1}.g^{-1}$, lactate release by 17.0 ± 2.9 $\mu mol.h^{-1}.g^{-1}$ and perfusion pressure by 68 ± 15 mmHg above basal ($n = 5$), all values corresponding to those obtained when one hindlimb was perfused. In addition, a quantity of erythrocytes was observed upon infusion of NE.

A dose of 1 μM 5-HT decreased $\dot{V}O_2$ by 3.7 ± 0.2 $\mu mol.h^{-1}.g^{-1}$ (50 ± 4 %) and increased perfusion pressure by 66 ± 7 mmHg (329 ± 28 %) above basal values, neither proportion significantly different from experiments where one hindlimb was perfused.

Constant infusion of FITC-dextran (10 $\mu g.ml^{-1}$ final) had no effect on perfusion pressure or $\dot{V}O_2$, and had reached plateau fluorescence within 10 min. In hindlimbs where 0.1 μM NE was infused for 10 min in the presence of FITC-dextran and subsequently reinfused $t = 6$ min after FITC-dextran removal (according to protocol *I*), a transient increase in relative intensity was observed in comparison to control (Figure 7.3 *A*). The difference between the two curves transformed to an entrapped volume of 220 ± 19 μl ($n = 4$, Figure 7.4). When t was increased to 15 min the entrapped volume decreased to 85 ± 5 μl ($n = 2$, Figure 7.4). When 0.1 μM NE was infused according to protocol *II* ($t = 6$ min), the entrapped volume was found to be negligible (data not shown).

In hindlimbs where 1 μM 5-HT was infused in the presence of FITC-dextran and not removed until $t = 6$ min after FITC-dextran removal (according to protocol *II*), the relative intensity increased slightly and remained elevated for approx. 14 min in comparison to control (Figure 7.3 *B*). The difference between the two curves transformed to an entrapped volume of 105 ± 13 μl ($n = 4$, Figure 7.4). When t was increased to 15 min the volume decreased to 50 ± 10 μl ($n = 2$, Figure 7.4). When 1 μM 5-HT was infused according to protocol *I* and $t = 6$ min, the entrapped volume was found to be negligible (data not shown).

The total vascular volume of untreated hindlimbs was 2.36 ± 0.04 ml ($n = 4$, Figure 7.3 *C*). As the entrapped volume by both NE and 5-HT appeared to be time dependent, suggesting a component of slow washout (Figure 7.4), the estimation of entrapped volume at the point of FITC-dextran removal was obtained by extrapolation of the curves to $t = 0$ min. Exponential curves were fitted for each

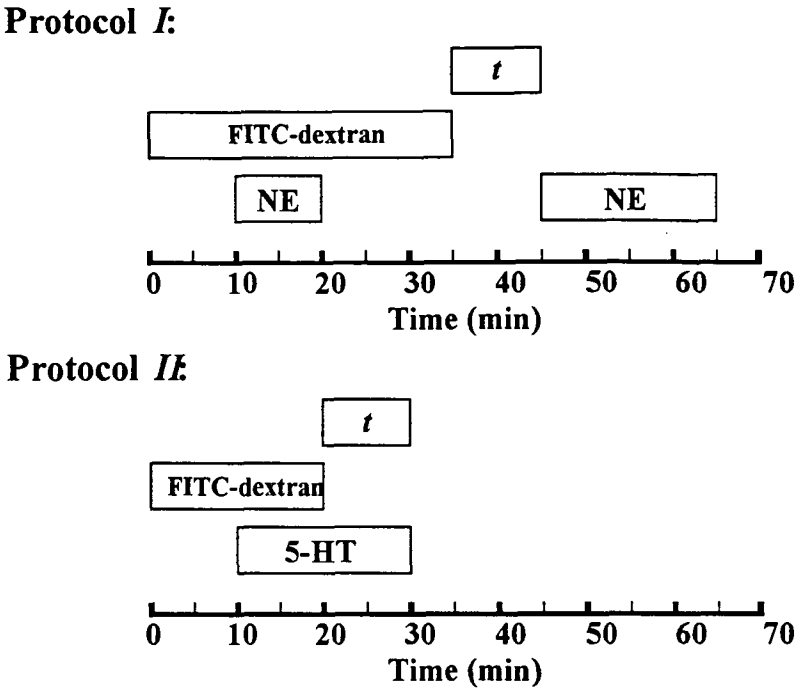

Figure 7.2 Perfusion protocol for determining the entrapment of FITC-dextran during infusion of NE or 5-HT.

NE (0.1 μM), 5-HT (1 μM) or FITC-dextran (10 $\mu g.ml^{-1}$) were infused for the time indicated by bars. The period indicated by *t* varied between experiments (see Figure 7.4), and in this example was 10 min. Venous effluent samples were intermittently collected to measure relative intensity throughout the periods of FITC-dextran infusion, and then more frequently from *t* = 0 min, the time of FITC-dextran removal.

Protocol *I*: Removal of the agonist until basal $\dot{V}O_2$ and perfusion pressure were obtained (15 min) then cessation of FITC-dextran infusion. After a defined period (*t*) the agonist was reinfused and measurement of washout of entrapped marker commenced; or

Protocol *II*: Cessation of FITC-dextran infusion after a defined period (*t*) before removal of the agonist and measurement of washout of entrapped marker.

FITC, fluorescein isothiocyanate; NE, norepinephrine; 5-HT, serotonin.
Modified from Newman (1992).

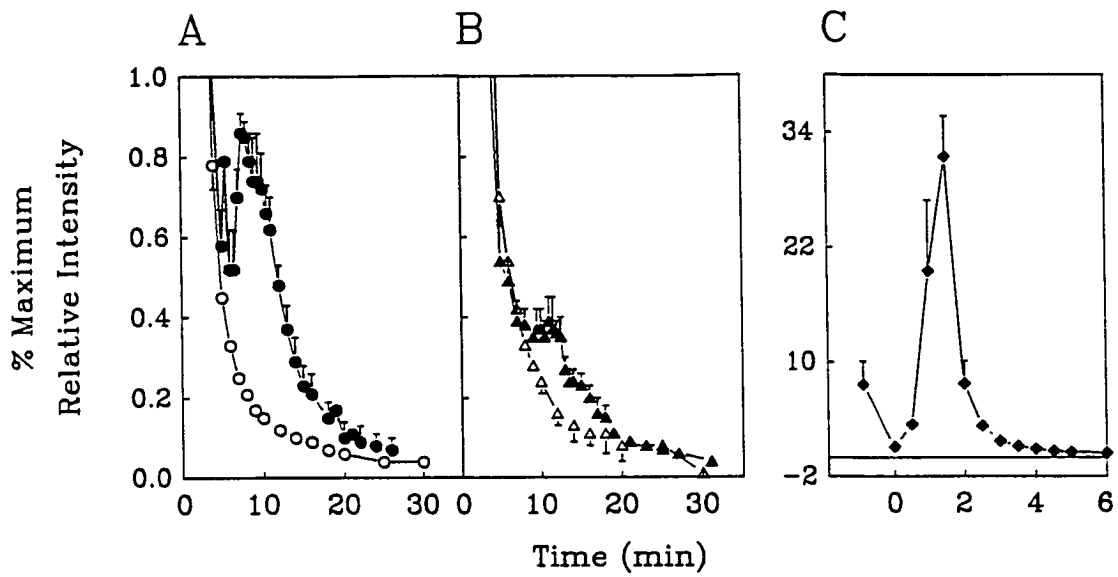

Figure 7.3 Time course of the washout of NE- and 5-HT-mediated and total entrapped spaces in perfused hindlimb.

Both hindlimbs of rats were perfused at 8 $ml.min^{-1}$ as described in Figure 7.3 and 10 $\mu g.ml^{-1}$ FITC-dextran was infused for 10 min to reach equilibrium. The data are expressed as a percent of the maximum relative intensity. Panel *A* followed protocol *I* and shows the washout of FITC-dextran when NE was (●, 0.1 μM, t = 6 min) or was not (○) reinfused. Panel ***B*** followed protocol *II* and shows the washout of FITC-dextran when 5-HT (▲, 1 μM, t = 6 min) or was not (△) removed. Panel *C* shows the washout of trapped FITC-dextran from untreated hindlimbs (◆) once the inflow and outflow tubing was devoid of marker. Time = 0 min refers to the removal of FITC-dextran (t = 0, ***A*** & ***B***) or the reconnection of flow to the rat (*C*). Values are means ± SE for 4 hindlimbs. When not visible, error bars are within symbols. FITC, fluorescein isothiocyanate; NE, norepinephrine; 5-HT, serotonin.
Modified from Newman (1992).

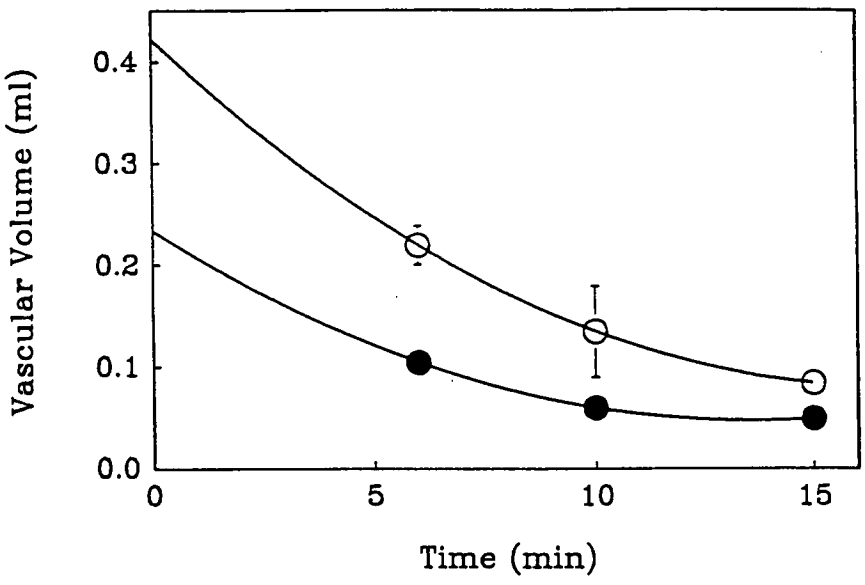

Figure 7.4 Effect of time on the apparent volume entrapped by NE and 5-HT in perfused hindlimbs.

Both hindlimbs of rats were perfused at 8 $ml.min^{-1}$ as described in Figure 7.3. The perfusion protocol was as given in Figure 7.2, where NE (O, 0.1 μM) and 5-HT (●,1 μM) were infused as described by Protocols *I* and *II*, respectively, and *t* was increased from 6 min to include data for 10 and 15 min. The data were calculated to represent volumes as described in Section 7.2.2. Exponential equations were iteratively fitted using EXPFIT (Guardabasso *et al.*, 1991) and extrapolated to time = 0 min, the point when FITC-dextran was removed. Values are means ± SE for 4 hindlimbs for $t = 6$, and 2 hindlimbs each for $t = 10$ and 15 min. When not visible, error bars are within symbols. FITC, fluorescein isothiocyanate; NE, norepinephrine; 5-HT, serotonin.
Modified from Newman (1992).

treatment with r values of 0.977 and 0.915 for NE and 5-HT, respectively. The extrapolated volumes were 0.42 ml for NE and 0.18 ml for 5-HT (Figure 7.4). Thus the total basal vascular volume accessed by NE was approx. 18% and that closed off by 5-HT was 8%.

7.3.3 Microsphere injection.

The size distribution of the injected microspheres is given in Figure 7.5 (*A*). The major proportion of spheres were between 8 and 16 μm in diameter. In control experiments, only 0.7 ± 0.3 % (n = 3) of injected microspheres were observed in the venous effluent, whereas in 5-HT-treated hindlimbs, a greater proportion (1.9 ± 0.4 %, n = 3) of injected spheres were recovered ($P < 0.05$, unpaired Student's t test). Of the spheres that were recovered, the distribution was not significantly different between control and 5-HT treated groups, although more 7.25 μm spheres were observed in 5-HT treated hindlimbs compared to control (Figure 7.6). With either treatment, few spheres greater than 15 μm were observed in the venous effluent.

7.4 Discussion.

The purpose of this study was to determine whether the observed changes in hindlimb metabolism induced by the vasoconstrictors NE and 5-HT under constant-flow perfusion conditions could be explained by changes in flow distribution within the rat hindlimb vasculature. The studies were performed in the absence or presence of vasoconstrictors and were determined by three methods. Firstly, efflux kinetics of bolus injected Evans blue dye were compared between control, NE- or 5-HT-treated and flow-restricted hindlimbs. Secondly, fluorescein-labelled dextran was trapped in the absence or presence of NE or 5-HT and subsequent washout was monitored. This method also allowed an estimation of trapped volume compared to total basal volume. Finally, unlabelled microspheres over a wide size distribution were injected in the absence or presence of 5-HT, and the effluent washout numbers were counted and compared.

The dye experiments suggested that 75 nM NE significantly altered the distribution of flow through the hindlimb in conjunction with the increases in perfusion pressure and $\dot{V}O_2$. In each experiment the effect of NE was to slightly lower the maximum absorbance of dye washout in comparison to basal. This, in

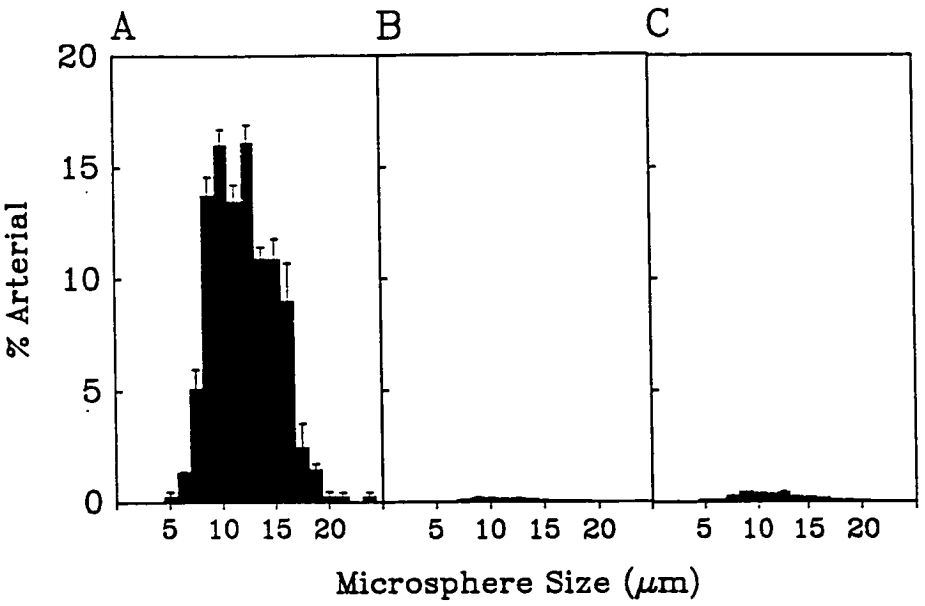

Figure 7.5 Effect of 5-HT on the washout of injected microspheres in perfused hindlimb I.

Hindlimbs from 180-200 g rats were perfused at 25°C and a constant flow rate of 4 $ml.min^{-1}$ with perfusion medium containing 2% bovine serum albumin and gassed with 95% O_2-5% CO_2. Panel *A* shows the size distribution of microspheres injected into the arterial perfusion line. Venous effluent spheres were collected and counted in the absence (Panel *B*) and presence (Panel *C*) of 5-HT (1 μM) and expressed as a proportion of the total number of spheres injected into the arterial line. Values given are means ± SE for three perfusions. 5-HT, serotonin.

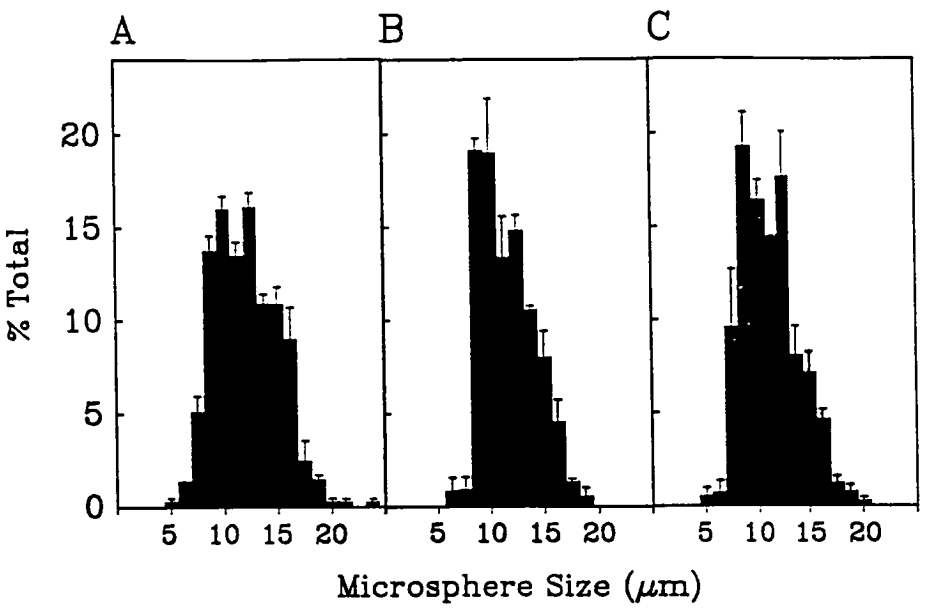

Figure 7.6 Effect of 5-HT on the washout of injected microspheres in perfused hindlimb II.

Data obtained in Figure 7.6 are expressed as a percent of the total number of spheres counted for each treatment. Panel *A* shows the size distribution of microspheres injected into the arterial perfusion line. Venous effluent spheres were collected and counted in the absence (Panel *B*) and presence (Panel *C*) of 5-HT (1 μM). Values given are means ± SE for three perfusions. 5-HT, serotonin.

addition to the observed washout of a small number of erythrocytes, tends to suggest NE is in some way causing a small portion of the flow to reach previously unperfused vessels.

The entrapment studies involved perfusion of both hindlimbs. In general, the effects of NE were consistent with those obtained when one hindlimb was perfused. NE increased $\dot{V}O_2$, lactate and erythrocyte release in association with vasoconstriction. The results supported the dye washout studies in that NE-induced vasoconstriction was shown to recruit a space that was only perfused slowly in the absence of constriction. It has already been discussed that NE did not appear to increase flow to hypoxic regions (see Chapter 1). If smooth muscle work does not account for the large increases in metabolism during vasoconstriction, a form of agonist-mediated uncoupling or futile cycling must be postulated such that the responses were sustained, reproducible and independent of skeletal muscle contraction-mediated metabolism. Thus the increase in perfused volume in the presence of NE may suggest heterogeneity of microvascular flow at the level of the capillaries, which did not compromise oxygenation of the surrounding skeletal muscle. Alternatively, the entrapped space may be in relatively metabolically inactive regions such as bone or connective tissue and changes in flow not related to changes in metabolism. This possibility could be explored by trapping and detecting either fluorescent or radioactive markers in firstly whole, then sectioned hindlimb tissue. For example, Bassingthwaighte and co-workers used the soluble, radioactively labelled flow marker 2-iododesmethylimipramine to determine regional flow in sectioned rabbit (Bassingthwaighte *et al.*, 1987) and sheep (Bassingthwaighte *et al.*, 1990) hearts.

Because of the large pressures that would be involved, no experiments were performed to determine possible changes in flow induced by high doses of NE (2.5 μM, Chapter 2).

In contrast to the effects of NE, 5-HT appeared to decrease the perfusion space of the hindlimb. The dye injection studies showed that 5-HT appeared to significantly alter the distribution of flow in the hindlimb, in a manner opposite to that of NE yet similar to that observed when the hindlimb was reversibly occluded below the knee. Maximum absorbance values in the venous effluent were significantly higher than those in the control condition indicating a proportion of dye had a shorter path length through the hindlimb. The extent of vasoconstriction was similar in both the NE and 5-HT experiments yet the effects on the absorbance profiles were quite

different.

The FITC-dextran entrapment studies supported the dye efflux studies in that 5-HT appeared to decrease the perfused region. The volume of FITC-dextran entrapped by 5-HT appeared to decrease with time, and comprised less than 10% of the basal volume, again apparently less than the percent decrease in $\dot{V}O_2$. The results do not preclude the possibility of altered distribution of flow in the presence of 5-HT. 5-HT may reduce, but not deny, flow to metabolically active regions.

One consideration that must be made with the two indicator techniques is that heterogeneities in transit time and washout volumes are the composite of heterogeneities in both flow and volume. Thus if the hormones decreased the total vascular volume (by decreasing the radius of blood vessels) during vasoconstriction, the differences in transit time would be a combination of increased velocity (to maintain constant flow) and alterations in the number of blood vessels perfused. In addition, entrapped FITC-dextran volumes do not give an indication of the number of capillaries or the radius of the vessels the dextran was trapped in. Thus the small proportion of trapped volume observed with 5-HT could represent a large change in number of perfused capillaries or a small change in the number of large vessels perfused. However it must also be considered that a heterogeneity of flow may not underlie the observed metabolic effects during vasoconstriction. Possible mechanisms to increase $\dot{V}O_2$ that are not dependent on changes in flow pattern include an endothelial cell shear-stress-mediated trigger of paranchymal metabolism (Chapter 1, Section 1.3.2.3) or vascular thermogenesis (Chapter 1, Section 1.3.2.4).

The lack of marked difference in the washout pattern of injected microspheres in the presence of 5-HT compared to control suggests 5-HT was not opening direct anatomical arterio-venous (A-V) shunts (>15 μm) in skeletal muscle. The existence of such vascular bypasses in skeletal muscle has been debated, with functional evidence to support their presence (Lopez-Majano *et al.*, 1969) but in general refuted on morphological grounds (Rhodin, 1977), although A-V anastomoses are reported to be common in the surface fascia of feline tenuissimus muscle (Eriksson & Myrhage, 1972) and in the fascia covering the feline cremaster muscle (Grant & Wright, 1968) and within hindlimb skeletal muscle of rabbits (Warren & Ledingham, 1974). Indeed, Lindbom and Arfors (1984) noted that constriction of transverse arterioles may close their lumen, stopping flow to nutritive capillaries, but flow would continue through arcade arterioles into adjacent non-nutritive connective tissue vessels. In rat spinotrapezius muscle, the size of these

arcade arterioles ranges between 20-50 μm, and the root of the transverse arterioles between 8-30 μm (Zweifach *et al.*, 1981, Figure 8.1).

A recent study by Harrison *et al.* (1990a) on local oxygen supply and blood flow regulation in contracting muscle in dogs and rabbits suggested the presence of high-flow capillaries (shunts or non-nutritive capillaries) that although only 13% of the capillary density (Potter & Groom, 1983) could carry approx. 70% of the flow under resting conditions and be adjusted by local regulatory mechanisms when oxygen demand was increased (i.e. in exercise). Using the data of Potter and Groom (1983) Harrison *et al.* (1990a) postulated the diameter of these putative shunts would be 7-8 μm compared to the nutritive capillaries of 4-6 μm diameter. Thus alterations in the proportion of flow between the two types would not be detectable with the commonly used 15 μm spheres (Laughlin *et al.*, 1982; Piiper *et al.*, 1985; Gorski *et al.*, 1986; Iversen & Nicolaysen, 1990; Bassingthwaighte *et al.*, 1990) or the majority of spheres used in this study. Thus in this study, the lack of marked increases in effluent microspheres in the presence of 5-HT suggests that either the A-V shunt vessels had small luminal diameters or that most of the perfusate flow still passed through capillaries that were less nutritive.

CHAPTER 8

Thesis discussion.

8.1 Summary of findings.

The main aim of this thesis was to characterize the vascular and associated metabolic effects of norepinephrine (NE) and serotonin (5-HT) in the constant-flow perfused rat hindlimb. In the course of this work, three important findings have emerged:

(1) Separate α_1-adrenoceptors (ARs) control NE-mediated vasoconstriction-associated increases and decreases in hindlimb metabolism;

(2) The fuel and extracellular Ca^{2+}-dependency of smooth muscle constriction is related to the control of oxygen, substrate and hormone delivery to resting and active tisssue; and

(3) Vascular site-selective receptor gene expression may have a physiological role.

The hormones NE and 5-HT proved to be interesting vasomodulators that had heterogeneous effects on hindlimb metabolism, apparently due to effects at different levels of the vascular tree. The vasoconstriction-associated effects on metabolism were observed in perfused, but not incubated, preparations thereby underlining the importance of a functional vascular system in the control of metabolism.

Vasoconstriction mediated by low doses of NE occurs through a subgroup of α_1-ARs and is associated with large increases in hindlimb metabolism. These effects are selectively opposed by β-AR and "5-HT_1-like" agonists (Chapter 2), which is consistent with an action at sites within the microcirculation, perhaps at A3 or A4 arterioles (Figure 8.1). Further support for these sites being involved comes from the susceptibility of low-dose NE-mediated constriction to inhibition by hypoxic conditions and by removal of extracellular Ca^{2+}. Both these events are usually associated with constriction of small arterioles (Chapters 3 & 4). There is an overall increase in α-AR density with decreasing vessel size, and perhaps an increased number/affinity of ARs in fast-twitch glycolytic fibre types (introduced in Chapter 1).

The large increase in hindlimb metabolism observed upon infusion of low-dose NE may be due to the work done during constriction of the relatively large number of small arterioles as reviewed by Colquhoun and Clark (1991). The possibility that NE-mediated constriction altered the microcirculatory flow pattern was addressed (Chapter 7), and the results could also support the alternative concept of a flow redistribution to capillaries feeding tissues which are triggered to increase their metabolism by an as yet undetermined mechanism. Both these alternatives are receiving ongoing study, and are not necessarily mutually exclusive.

The possibility that skeletal muscle has the potential to increase its metabolism, and this increase is controlled by circulating hormones and neuronally-released factors is an important observation and may improve our understanding of thermoregulation. However, thermoregulation may not be as simple as an on-off positive thermogenic mechanism. In situations such as long-term overeating (obesity), positive thermogenic mechanisms may need to be overridden to prevent overheating and to maintain vital functions. The main finding from this work may be related to this latter situation and demonstrates how control of blood flow by vasoconstrictors can also decrease metabolism in skeletal muscle. This finding adds functional significance to the existence of receptor subtypes in discrete vascular locations.

As outlined in Chapter 1, sympathetic nervous system (SNS) efferent activity increases to skeletal muscle postprandially, when the body is exposed to cold or hypoxia, and during intense muscular exercise. Depending on the intensity of each situation, there may be a necessity for reduced flow to the energetically-expensive skeletal muscle bed. Thus it is proposed that the locus of flow control shifts to larger vessels, probably at discrete sites, and the delivery of nutrients to either resting or contracting muscle can be markedly reduced, thereby temporarily compromising tissue metabolism. In this study it was found that these sites probably express 5-HT_{2A}-receptors and α_{1B}-ARs. Results from Chapters 3 and 4 showed both 5-HT- and high-dose NE-mediated vasoconstriction could be supported anaerobically and in the absence of extracellular Ca^{2+}, thus providing a method of dissociating positive and negative thermogenic vasoconstrictors. These latter findings are consistent with constriction of larger vessels.

As reviewed in Chapter 2, literature reports generally support a 5-HT-mediated constriction of larger vessels. In a skeletal muscle preparation, Alsip and Harris (1991) reported that 5-HT caused a 5-HT_2-receptor-mediated constriction of

large (A1) arterioles and a orphan 5-HT-receptor dilation of small (A3 or A4) arterioles (consistent with results in Chapter 2). The result of constriction at these sites was an overall decrease in resting (Chapters 2-7) and contracting (Chapter 6) hindlimb metabolism despite constant-flow. Thus it is possible that vigorous constriction of arteries or larger arterioles (A1, Figure 8.1) by 5-HT may divert flow through functional, but not anatomically-typical arterio-venous, vascular shunts in the hindlimb (Chapter 8). This may occur as a result of an alteration in the distribution of flow to different muscle groups (possibly away from slow-oxidative and more to fast-glycolytic) and/or by selective reduction or denial of flow to the microvasculature within a muscle (Figure 8.1). In either case, oxygen and substrate availability is reduced to active smooth and skeletal muscle cells.

A similar proposal is also put forward for high-dose NE. The α_1-AR subgroup (presumably α_{1B}, Chapter 4) stimulated may be located on vessels upstream from the α_1-ARs stimulated by low doses of NE, probably at the transverse arteriole roots. These vessels are reported to have the highest tone *in vivo* (Schmid-Schönbein *et al.*, 1987), have dense sympathetic innervation (Saltzman *et al.*, 1992) and can readily close their lumen (Schmid-Schönbein & Murakami, 1985). Constriction at these sites could be the principal control mechanism for modulating the capillary perfusion (Saltzman *et al.*, 1992).

8.2 Implications of findings.

In the light of these findings it is proposed that resting skeletal muscle contributes to whole body thermogenesis and that it is controlled by total, and well as zonal (within muscle), nutrient delivery. The fine balance that must exist between delivery and usage of nutrients in skeletal muscle and without compromizing function in other parts of the body is controlled, at least in part, by the release of vasoactive agents from the sympathetic nerve endings. Interruptions to this balance could markedly alter skeletal muscle metabolism. Elevations in SNS activity observed in disorders such as hypertension and obesity may eventually lead to significant alterations in skeletal muscle blood flow and thereby metabolism. Reductions in the delivery of oxygen, fuels and hormones (including insulin) to muscle could contribute to the onset and presence (rather than be an effect) of the disorders, including obesity, non-insulin-dependent diabetes and atherosclerosis, often associated with hypertension (Figure 8.2). The clinical occurrence and importance of this possibility is currently receiving attention, and has recently been reviewed by Baron *et al.* (1993) and Julius *et al.* (1992).

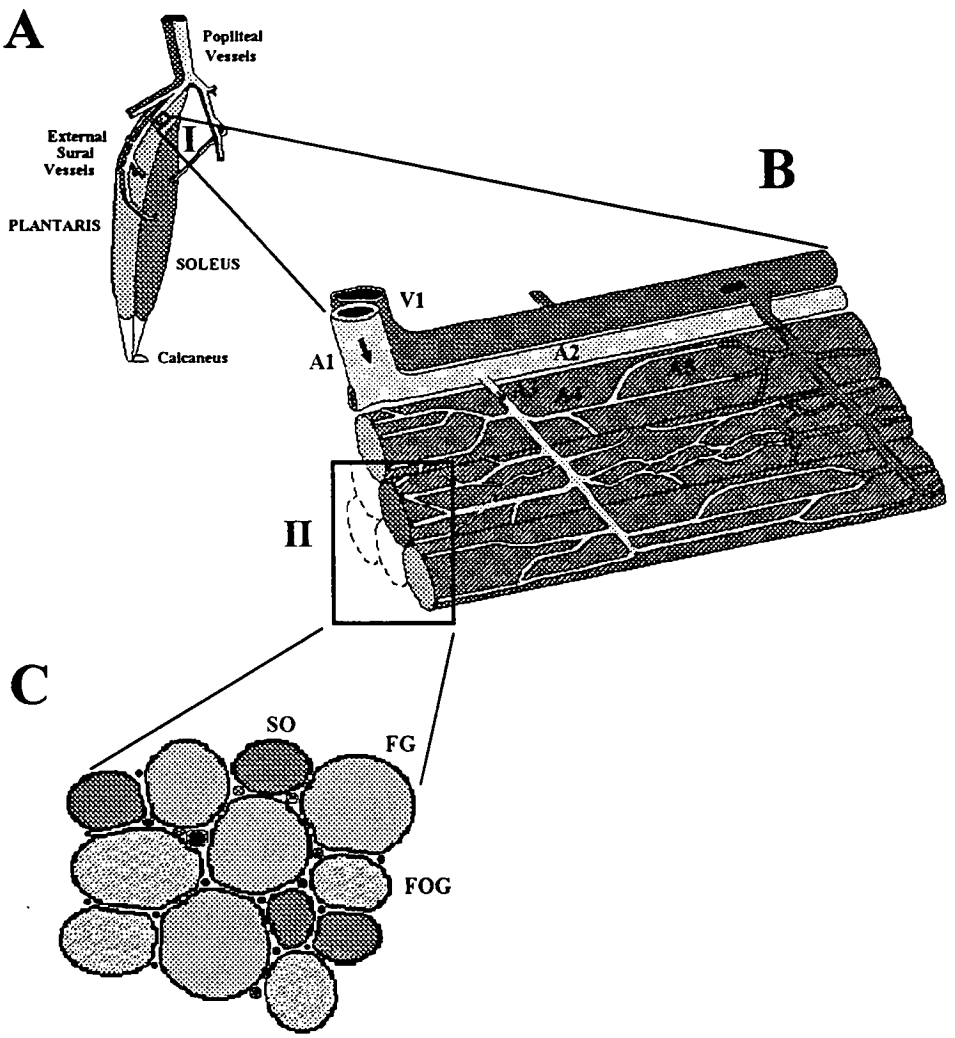

Figure 8.1 Schematic diagram of the branching sequence in the vascular network of skeletal muscle.

Panel **A** shows the external sural artery feeding the soleus and plantaris muscles of the lower hindlimb. Panel **B** is an enlargement from within BOX **I**; and arterial branchings are numbered beginning with the sector that feeds the arteries: **A1**, feeding artery (60-120 μm); **A2**, arcade arteriole (20-50 μm); **A3**, transverse arteriole (8-30 μm); **A4**, precapillary arteriole (4-12 μm); and **A5**, capillary. Panel **C** is a transverse section of BOX **II**, and shows a typical arrangement of the slow-twitch oxidative (**SO**) fast-twitch oxidative/glycolytic (**FOG**) and fast-twitch glycolytic (**FG**) fibre types within the plantaris muscle. Modified from Eriksson and Myrhage (1972), Zweifach *et al.* (1981), Sullivan and Pittman (1982) and Saltzman *et al.* (1992).

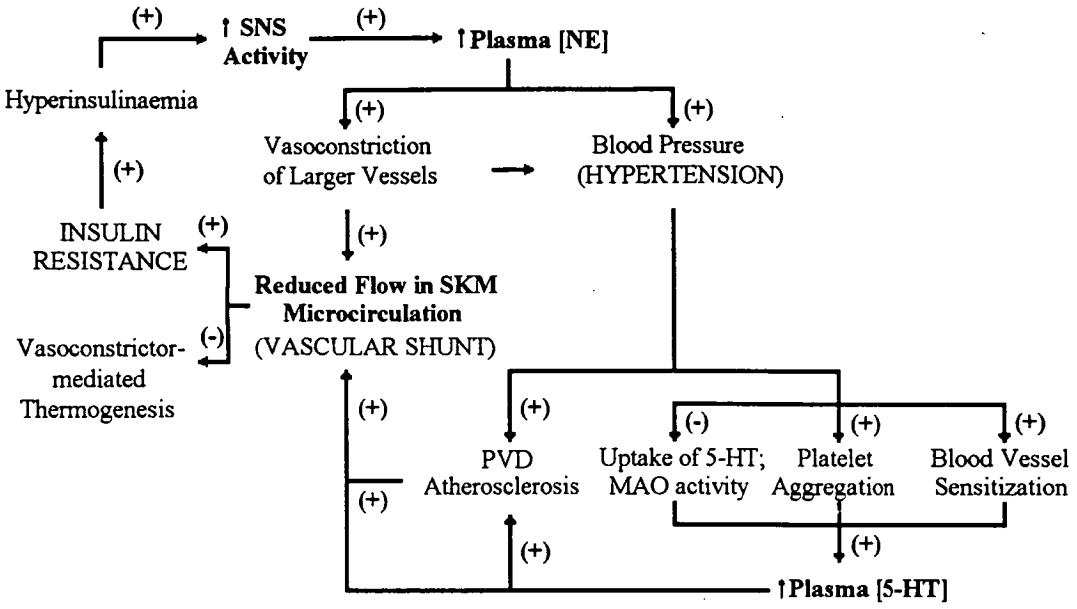

Figure 8.2 The link between hypertension and insulin-resistance in SKM: A model.

This model depicts the possible causes and consequences of reduced skeletal muscle (SKM) blood flow. It should be considered in conjunction with the modified Landsberg hypothesis presented in Chapter 1 (Figure 1.1). In situations where the sympathetic nervous system (SNS) is over-stimulated, for example in obese patients, the plasma levels of norepinephrine (NE) and serotonin (5-HT) are increased, causing vasoconstriction of large, feeding or root arterioles through α_{1B}-adrenoceptors and 5-HT_{2A}-receptors, respectively. This constriction reduces SKM nutritive microcirculation blood flow, impairing nutrient (eg. O_2, fatty acid and glucose) and hormone (eg. insulin) delivery. Long-term increases in blood pressure (hypertension) are often associated with peripheral vascular disease (PVD) and atherosclerosis, which also contribute to the reduction in nutrient access. This results in a decrease in vascular thermogenesis and causes a state of insulin resistance, which may, in turn, stimulate SNS activity thereby completing the cycle.

Modified from Vanhoutte and Cohen (1983); Landsberg (1986); Clark *et al.* (1991); and Barradas and Mikhailidis (1992). MAO, monoamine oxidase.

8.3 Future considerations.

The location of agonist-mediated responses warrants future investigation. The most direct approach would be to use cDNA clones of the various α_1-ARs and 5-HT_2-receptors. Once the presence of the mRNAs was confirmed, *in situ* hybridization with cDNA probes or with fluorescent of radiolabelled antibodies to the receptor subtypes should localize receptor populations.

Another important set of experiments would be to determine whether the effects observed *in vitro* are relevant to *in vivo* situations. Constant-pressure perfusions at more physiological levels using medium containing erythrocytes would allow an assessment of changes in flow and associated changes in metabolism upon infusion of agents.

These problems could be partially addressed by visualizing the effects of vasomodulators *in situ* by use of intravital microscopy. This procedure has drawbacks in that access is limited to surface, predominantly fast-twitch glycolytic fibre-type muscle groups and only their surface microcirculatory networks. The microcirculation of two hindlimb muscle groups, the extensor digitorum longus (Tyml & Budreau, 1991) and tibialis (Lund *et al.*, 1987), have been successfully characterized *in situ*. However, to compare metabolism to blood flow distribution, a constant-flow erythrocyte-perfused preparation would appear more relevant to these studies.

The above experiments could also be repeated in various animal models. Both spontaneously hypertensive (Finch *et al.*, 1990) and fructose-fed (Hwang *et al.*, 1987) rats display insulin resistance. The altered responsiveness to vasoactive agents in these disorders (reviewed by Struyker-Boudier *et al.*, 1990) may demonstrate the importance of blood flow control in determining tissue metabolism.

In conclusion, manipulation of microvascular haemodynamics in obesity, diabetes and hypertension may provide a means of treating and protecting against the complications of these conditions. Drugs such as ketanserin may lower blood glucose by opposing both 5-HT- and NE-mediated vasoconstriction, thereby improving insulin and glucose access to skeletal muscle (Figure 8.2). Agents that selectively oppose stimulation of 5-HT_{2A}-receptors and especially α_{1B}-adrenoceptors may provide improved treatments for obese or non-obese patients with Type II diabetes and hypertension.

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